THE EFFECTS OF KETOCONAZOLE ON THE OXIDATION OF LOW DENSITY LIPOPROTEINS BY BOVINE AORTIC SMOOTH MUSCLE CELLS

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by

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An abstract of a Thesis by

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Bovine aortic smooth muscle cells (BASMC) in culture have been shown to oxidatively modify human low-density lipoprotein (LDL). Such oxidized LDL appears to be a significant factor in the etiology of atherosclerosis. This work shows that the azole anti-fungal drug ketoconazole when added to the culture medium of BASMC inhibits LDL oxidation. Confluent BASMC were fed 1 ml of F-12/DMEM containing LDL (320 μg protein). The cells over a 24 hour period caused a 2x increase in LDL oxidation as measured by an increase in thiobarbituric acid reactive substances. Copper at 5 μM enhanced by 2x the oxidation of LDL by BASMC. Oxidized LDL was found to be cytotoxic to the BASMC. Addition of ketoconazole to the cell medium caused a concentration dependent inhibition of LDL oxidation by BASMC. Maximum inhibition of LDL oxidation was achieved at 20 μM of ketoconazole. BASMC viability was maintained in the presence of ketoconazole and LDL as determined by the protein content per well.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

INTRODUCTION

At the present time, one of the leading causes of death in Western industrialized societies is atherosclerosis (1). In the United States, more than half the population has a level of circulating low-density lipoprotein (LDL) that puts them at risk for developing atherosclerosis. High levels of LDL contribute to the development of plaques in the wall of arteries, which can inhibit blood flow enough that a clot forms, obstructing an artery and causing a heart attack or a stroke (1).

The atherosclerotic plaques that cause heart attacks or strokes develop slowly (1). The thin layer of endothelial cells lining the interior of the artery are damaged (i.e., several cells are lifted up by the flow of blood being split at a branching in the vessel), initiating the formation of a plaque. LDL particles and blood platelets are able to penetrate into the damaged and leaky endothelium layer. Hormones such as platelet-derived growth factor are released into the endothelium, stimulating smooth-muscle cells in the layer below the endothelium to multiply and migrate into the damaged area. Concurrently, circulating monocytes are attracted to the damaged site, invade it, and are stimulated to become scavenger macrophages. The smooth muscle cells and macrophages ingest and degrade (oxidize) the LDL, and become lipid-laden foam cells. Studies using a variety of cultured cells (monocytes/macrophages, endothelial, and smooth muscle cells) have suggested that LDL must first be modified (oxidized) in some way to augment its uptake into cells in order to produce the massive cholesterol accumulation seen in atherosclerotic plaques (2). Cellular uptake of LDL may be accelerated by the cell-mediated oxidative modification of LDL. If the level of blood LDL is high, cholesterol derived from the LDL accumulates in and among the foam cells (lipid-filled smooth muscle cells and monocytes/macrophages). The accumulation of cholesterol, cells, and debris form an atheroma, which over time can enlarge and narrow the diameter of the artery, leading to thrombosis (1).

LDL is the major cholesterol carrier in the blood stream. It is a spherical particle with a mass of three million daltons and a diameter of 22 nanometers (1). Its core consists of approximately 1500 cholesterol esters (the cholesterol esters are each a cholesterol molecule attached by an ester linkage to a long fatty acid chain). The core is shielded from the plasma by a detergent coat consisting of 800 molecules of phospholipid, 500 molecules of unesterified cholesterol, and one large protein molecule, apoprotein *B*-100. The phospholipids of the coat are arranged so that their hydrophilic heads are outside, allowing the LDL to be dissolved in the blood or intercellular fluid (1).

The level of LDL circulating in the blood is determined by a number of factors. The most obvious one is an individual's diet. Diets high in cholesterol and saturated fats elevate serum LDL. Another determiner is the number of LDL receptors present. LDL receptors are found on the surfaces of cells, and they bind LDL particles and extract them from the fluid surrounding the cells (1,3). The LDL is taken into cells via the receptor and is broken down, giving cholesterol to serve the cell's needs. In supplying the cells with cholesterol, the receptors perform a second function important to the development/prevention of atherosclerosis -- removing LDL from the bloodstream. The more receptors for LDL an individual has, the lower their levels of serum LDL. The body is able to use circulating LDL as a cholesterol source instead of having to manufacture its own (1).

The oxidative modification of LDL is a free radical process in which the LDL is depleted of its antioxidant capabilities and the polyunsaturated fatty acids are degraded by lipid peroxidation (4,5,6). Metal ions like copper and iron (6), or cells plus trace amounts of these metals can oxidize LDL. When LDL is incubated with cells, specifically smooth muscle cells, the LDL particle is modified. The polyunsaturated fatty acids in the LDL are peroxidized, which causes an increase in the density of the LDL particle (5). Possible causes of this modification of the LDL by the BASMC are hydrolytic enzymes (used by

The oxidation of LDL can, however, be inhibited by the use of antioxidants or metal chelating agents (2). Antioxidants such as butylated hydroxytoluene (BHT) and vitamin E have been shown to inhibit the oxidation of LDLs (2,5). Also of interest are agents that inhibit cytochrome P-450 enzymes, some of which can release activated O_2 that can then participate in the oxidation of LDL (2,7). One known inhibitor of cytochrome P-450 is Ketoconazole (KC).

Ketoconazole is currently marketed as an anti-fungal agent, and is a known cytochrome P-450 enzyme inhibitor (7). KC is an imidazole derivative with a number of clinical applications, including interfering with adrenal steroid hormone and testosterone biosynthesis (it is sometimes used in the treatment of prostate cancer and Cushing's disease) (7). It was originally marketed as a broad-spectrum oral anti-fungal agent, but when it was noted that patients receiving the drug showed lower levels of serum LDL, the idea of using KC to treat patients with elevated serum LDL (atherosclerosis) was posed.

It was the purpose of the present study to describe the growth characteristics and oxidative properties of a previously undocumented bovine aortic smooth muscle cell (BASMC) line; to determine the rate of these cells' oxidation of human LDL, and what factors will affect their oxidation of the LDL; to examine the possibilities of Ketoconazole as an inhibitor of the cellular and trace metal oxidation of human LDL; and to determine KC's effects (cytotoxicity, etc.) on the new line of BASMCs. In a series of experiments, KC was used in conjunction with tissue cultures of BASMC, along with the addition of trace metals, to determine its (KC) effectiveness at preventing the oxidation of LDL.

MATERIALS

The bovine aortic smooth muscle cells used were from a line obtained from S. Parthasarathy (University of California, San Diego, 92094). Ham's F-12/DMEM medium and fetal calf serum (FCS) were obtained from GIBCO (Grand Island, New York). Non-

obtained from Sigma Chemical Company (St. Louis, Missouri). Tissue culture plates were obtained from Sarstedt (Newton, N. C.). Common chemicals were obtained from Fisher (Pittsburgh, PA), or Sigma Chemical Company.

METHODS

Cells

BASMC were grown in Ham's F-12/DMEM medium + 10% FCS + 5% PenStrep + 5% NEAA (growth medium) in a humidified incubator with 5% CO_2 atmosphere at 37° C. For experiments, the BASMC were seeded on 6-well 9.5 cm^2 plates at a concentration of 5 x 10^5 cells/well, or in 24-well 2 cm^2 plates at a concentration of 1 x 10^5 cells/well. The cells were grown to confluence (4-6 days). At confluency (Day 0), the cells were rinsed with phosphate buffered saline (PBS, 100 mM KH_2PO_4 and 0.154 M NaCl, pH 7.4) to remove any traces of FCS, and treatment medium (medium without FCS) was added. The treatment substances, at the indicated concentrations, were added directly to the cells' medium. Controls consisted of treatment medium without cells.

Collection of Samples

At indicated times (0, 24, 48 hours), samples of cells or treatment medium were collected. The medium samples were collected and placed in tight- sealing microfuge tubes containing 10 µl of 20 mM BHT and 10 µl of 100 mM EDTA, Na_2 (to prevent further oxidation) (8). They were stored in the dark at -20° C until analysis by TBARs and/or electrophoresis (as described below). They consisted of the BASMC attached to the wells. Cell samples were rinsed with PBS, air-dried, and stored in the dark at 4° C until analysis by protein or viability assays (as described below).

Lipoprotein Preparation

LDL was prepared from human serum (HS) obtained from the Blood Bank of Iowa. Human LDL has a density of 1.063. The HS density was adjusted to 1.063 with

hours at 50,000 rpm, at 15° C in a Beckman L5 ultracentrifuge using a 70Ti rotor. The top layer of the solution (VLDL and LDL) was removed and added to a dialysis bag (3500 mw cutoff). The density of the VLDL/LDL solution was reduced to 1.006 by dialyzing against plasma density buffer (11.4 g/l NaCl + 0.13 g/l NaN_3) with 1mM EDTA added. The dialysis solution was changed twice during a 48 hour period. After dialyzing, the volume of VLDL/LDL was measured, and its density readjusted to 1.02 g/ml with KBr. Ultracentrifugation was repeated as above. The top layer (VLDL) was removed, the clear solution in the middle aspirated off, and the infranatant LDL pipetted out. The LDL (containing KBr, NaN_3, and EDTA) was stored in a tight-sealing tube under nitrogen gas.

Before each experiment, an aliquot of the concentrated LDL was adjusted to 16 mg protein/ml (protein content determined by BCA method described in Assays section) with PBS + EDTA (10 mM KH_2PO_4 + 3 mM EDTA, pH 7.4). The LDL was then dialyzed against 200 volumes PBS for 24 hours at 4° C in the dark to remove the EDTA and KBr. The PBS was made oxygen-free by vacuum degassing and nitrogen purging to protect the LDL from oxidation during dialysis, and it (PBS) was changed twice during the dialysis period (10). The LDL in the absence of EDTA was used in the LDL oxidation preparation. In experiments using LDL, the final LDL concentration was 320 µg/ml.

Cell-induced Modification of LDL

Cell-induced modification of LDL was performed by incubation of LDL with BASMC at 37° C for 24 to 48 hours. BASMC were grown to confluence in growth medium. The confluent cells were rinsed with PBS, 1 ml of treatment medium was added to the cells, and 20 µl of 16 mg/ml LDL was added to the medium on the cells (final LDL concentration of 320 µg/ml). As a positive control, oxidation was initiated with 10 µl of $CuSO_4$ (0.5 mM) in PBS, to make a final copper concentration of 5 µM (9,10,11). The progress of the reaction was followed by Thiobarbituric Acid Assay (TBARS).

Cell Photography

Light Microscopy. BASMC were seeded on 6-well 9.5 cm^2 plates and subjected to treatment as described above. Photos were taken of the cells in the medium containing the treatment substances (cells were not rinsed prior to photographing) using a 35 mm Nikon camera mounted to a Nikon inverted scope.

Electron Microscopy. BASMC were seeded in growth medium on 6-chamber, treated glass, tissue culture slides at a concentration of 5 x 10^5, and grown to confluence. At confluence (Day 0), the medium was aspirated off and the cells were rinsed with PBS. Treatment medium was added to the cells, along with the indicated treatment substances. After 24 hours, the cells were rinsed in a Koplin jar containing PBS. After 5 minutes, the slides were removed and fixed for 15 minutes in 5.5% glutaraldehyde. After fixing, the cells were rinsed with PBS, and post-fixed with 1% osmium tetroxide for 5 minutes (12). The cells were then rinsed with PBS, followed by a series of ethanol (EtOH) washes. The cells were soaked for 20 minutes in each of the EtOH washes (50%, 70%, 80%, 95%, and 100% EtOH), with the 100% EtOH wash repeated twice. The cells were then soaked in two washes of amyl acetate, each wash 10 minutes long. After the last amyl acetate wash, the cells were dried using a Ladd Critical Point Dryer, and then gold-coated using a Polaron Sputter Coater (Model 5100, Series II). The cells were scanned using a Hitachi S-500 Scanning Electron Microscope (13).

Assays

TBAR (Thiobarbituric Acid Reactive) Assay. The TBAR assay was used to measure the rate of oxidation. When the LDL is oxidized, it releases malondialdehyde (MDA), which reacts with thiobarbaturic acid. Results of the assay are given in nmoles MDA/ml, and the amount of TBAR material present is in proportion to the rate of oxidation.

Samples were assayed by adding 400 µl aliquot of sample to 1 ml of 20% trichloroacetic acid (TCA) and 1 ml of 1% thiobarbituric acid (TBA). The mixture was incubated at 95° C for 45 minutes, and then cooled in an ice water bath. About 1.5 ml of each sample was then transferred to a microfuge tube, and centrifuged at 13,600 x g for 5 minutes at room temperature. The supernatants' absorbencies were read at 532 nm using a Milton-Roy 1001 spectrophotometer. 1,1,3,3 tetramethoxypropane was used as a standard for MDA (8).

Protein Assay. The protein assay was the bicinchonic acid method as described by Pierce (Pierce, Rockford, IL). In an alkaline medium, protein reacts with Cu(II) yielding Cu(I). Bicinchonic acid reacts with the Cu(I) allowing for spectrophotometric determination of protein concentration. Results of the assay are given in µg protein/well.

The Standard Protocol by Pierce was followed. The Working Reagent was prepared by adding 50 parts of Reagent A to 1 part of Reagent B. BSA was used as the protein standard. The samples were collected from the wells by first rinsing each well with PBS, and then digesting the cells attached to the bottom with 1.0 ml of 0.1 M NaOH. Wells were scraped with a rubber policeman to ensure the digestion of all cellular material. 50 µl of each sample was added to the appropriate well in a 96-well, flat-bottom, microtiter plate. The BSA standards were added at 0, 5, 10, 15, 20, and 25 µl/well (QS each standard to 50 µl with H_2O). 150 µl of Working Reagent was added to each well (total volume in wells is 200 µl). The plate was covered and incubated at 37° C for 2 hours. The absorbance of each well was read at 562 nm using a Microtiter plate reader.

Agarose Gel Electrophoresis. Electrophoresis was used to give a visible representation of the extent of LDL oxidation. The greater the rate of LDL oxidation, the more the electrophoretic mobility of the LDL is changed, and the further the sample will move through the gel.

Agarose gel electrophoresis was performed using a Corning cell. A 1% gel was prepared by adding 0.2 grams agarose to 20 ml 1X buffer (0.05 M Tris/Tricine, pH 8.6). The agarose was poured onto Sigma electrophoresis film. 20 µl of sample mixture (80 µl sample + 20 µl of a 50% glycerol solution containing a trace amount of Bromophenol Blue) was added to appropriate slot on the gel. The gel was run in the same 1X buffer that the agarose was dissolved in, at 50 V for approximately 1.5 - 2 hours (until the Bromophenol Blue indicator is near the end of the gel). The gels were fixed in 2% TCA for 2 hours at room temperature (RT). The gel was then washed in distilled water for 60 minutes at RT. The gel was sandwiched between Whatman #3 filter paper, and dried using a hair dryer set on low speed, warm temperature. The dried gel was stained for lipid with 100 ml of Oil Red O at 37° C (in tissue culture incubator) for 24 hours. The gel was then rinsed for 5 minutes in distilled water, and allowed to air dry (8).

Cell Viability Assay (Hexosaminidase Reaction). The viability assay, used to estimate the number of cells present, used a chromogenic substrate (*p* -nitorphenol-*N* -acetyl-B-D-glucosaminide) for the cell lysosomal enzyme, hexosaminidase. Hexosaminidase is present in intact (live) cells. When the cells are lysed to release their hexosaminidase, and the substrate is added, a green color develops in proportion to the amount of enzyme present. The color absorbency was determined by a spectrophotometer. The results are expressed as number of cells/well.

The substrate for the enzyme hexosaminidase, *p* -nitorphenol-*N*-acetyl-B-D-glucosaminide, was dissolved at 7.5 mM in 0.1 M citrate buffer, pH 5. The solution was then mixed with an equal volume of 0.5 % Triton X-100 in water. 1000 µl of this mixture was added to each well containing cells, and incubated for 20 minutes at 37° C. The color reaction was developed and enzyme activity blocked by the addition of 50 mM glycine buffer, pH 10.4, containing 5 mM EDTA, 1300 µl/well. A 200 µl aliquot of each sample

The plate was read at 405 nm on a Microtiter plate reader. Cell numbers were found using a standard curve previously established through linear regression (9).

Ketoconazole (KC).

The KC was obtained from Janssen Pharmaceuticals. The working stock of KC was 1mM. The KC was dissolved in 95% EtOH, and was stored in the dark at 4° C. KC controls received an equal volume of 95% EtOH.

Statistics

Data were expressed as mean values plus or minus standard error of the mean (SEM). Data were subjected to the correlated groups t-test. Differences were considered significant when $P < 0.05$.

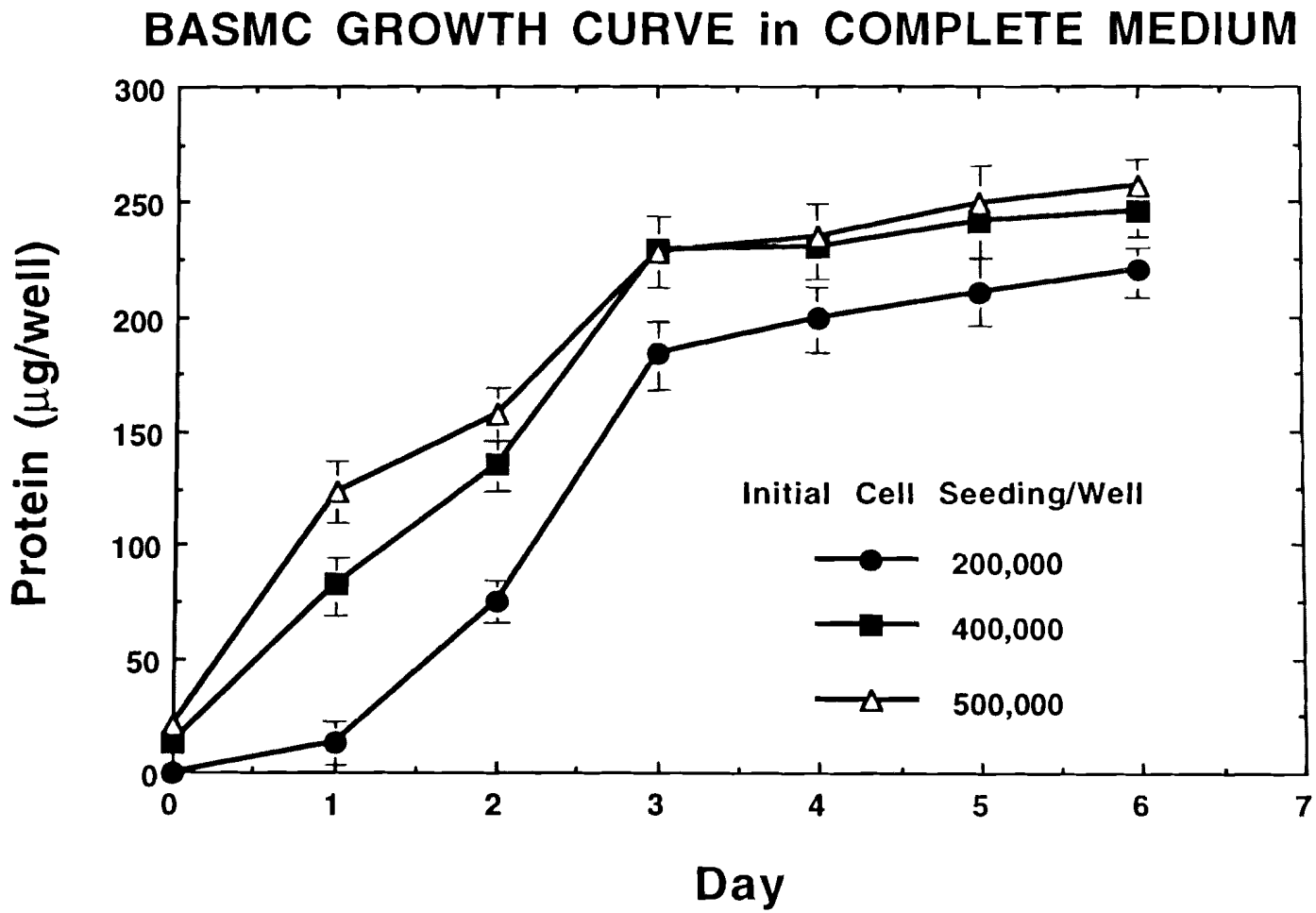
RESULTS

Cell Growth Characteristics

Before the initiation of any experiments, the growth pattern of the line of BASMC being used was determined. It was not known how long the cells had to grow before they reached confluence, or if the initial seeding concentration affected the rate of cell growth. The BASMC were seeded at various concentrations in growth medium and allowed to grow for 6 days (Figure 1). Cells were collected daily, and after the sixth day a protein assay was performed as described in the Methods section. It was found that by day 4, the initial seeding concentrations of 4 and 5 x 10^5 cells/well had reached confluence. Visual inspection of the cells through an inverted microscope confirmed the results given by the protein assay. By day 4, the cells appeared to be confluent. The edges of individual cells were in contact with the edges of neighboring cells (Figure 6 **A** and **B**), and there were no areas without cells ("bald" spots). The BASMC seeded at a concentration of 2 x 10^5 cells/well were still approaching confluence at day 6. Given another 2-3 days, these cells would probably reach confluence. The seeding concentrations of 4 and 5 x 10^5 cells/well

Figure 1. BASMC growth curve in growth medium.

On day 0, BASMC were seeded on 6-well 9.5 cm^2 plates at the indicated ıcentrations in growth medium. Each day cells were collected and the amount of protein · well was determined as described in the Methods section. Values represent the mean ± M of determinations from triplicate samples.


BASMC GROWTH CURVE in COMPLETE MEDIUM
Protein (µg/well)
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
Day
Initial Cell Seeding/Well
200,000
400,000
500,000

probably reached confluence together. Cell numbers are approximations, and when seeding cells on a new well, not all of the cells survive. Based on the seeding and growth curve data, it was decided that for future experiments, BASMC would be seeded at a concentration of 5 x 10^5 cells/well, and allowed to grow 4-6 days. This procedure ensured a confluent cell population with sufficient cells for experimentation.

Cell-Mediated Oxidation of LDL

Before any drug experiments could be performed, it needed to be established that the BASMC could oxidize LDL. The BASMC were seeded on 6-well 9.5 cm^2 plates in growth medium at 5 x 10^5 cells/well, and allowed to grow to confluence. At confluence the growth medium was removed, and the cells were re-fed treatment medium, to which LDL (320 µg/ml), $CuSO_4$ (5 µM), and LDL + $CuSO_4$ were added. $CuSO_4$ is a known oxidizer of LDL (5), and was used as a positive control for the initiation of oxidation. The results shown in Table 1 clearly show that BASMC increased the oxidation of LDL approximately 1.7 fold when compared to the auto-oxidation of LDL (LDL alone). $CuSO_4$ increased LDL oxidation by 4 fold over LDL auto-oxidation. When $CuSO_4$ was added to the BASMC, LDL oxidation increased 6 fold over cells and LDL. These results showed that $CuSO_4$ was a more efficient initiator of lipid peroxidation than BASMC.

Now that it was known that the BASMC could oxidize LDL, the next step was to determine if cell concentration affected the rate of LDL oxidation. BASMC were seeded on 6-well 9.5 cm^2 plates in growth medium at 1 x 10^5 cells/well, 2 x 10^5 cells/well, and 5 x 10^5 cells/well (Figure 2). The cells were allowed to settle. At 0 hour, the cells were fed treatment medium containing LDL (320 µg/ml). It was found that the rate of BASMC oxidation of LDL increases in proportion to the number of cells present. At the 24 hour point, cells seeded at 5 x 10^5 cells/well oxidized LDL approximately 2.8 fold that of 1 x 10^5 cells/well, and approximately 1.2 fold that of 2 x 10^5 cells/ml. The auto-oxidation of

Table 1. Effect of $CuSO_4$ on BASMC oxidation of LDL.

BASMC were seeded on 6-well 9.5 cm^2 plates at a concentration of 5 x 10^5 cells/well. The cells were grown to confluence (4-6 days) in growth medium. At confluency (0 hour), the cells were rinsed with PBS and fed treatment medium. The treatment substances (320 µg LDL/ml, and 5 µM $CuSO_4$) were added directly to the medium on the cells. After 24 hours, the medium was collected and aliquots analyzed for TBAR material as described in the Methods section. Values represent the mean of determinations from triplicate samples. SEM is given in parentheses.

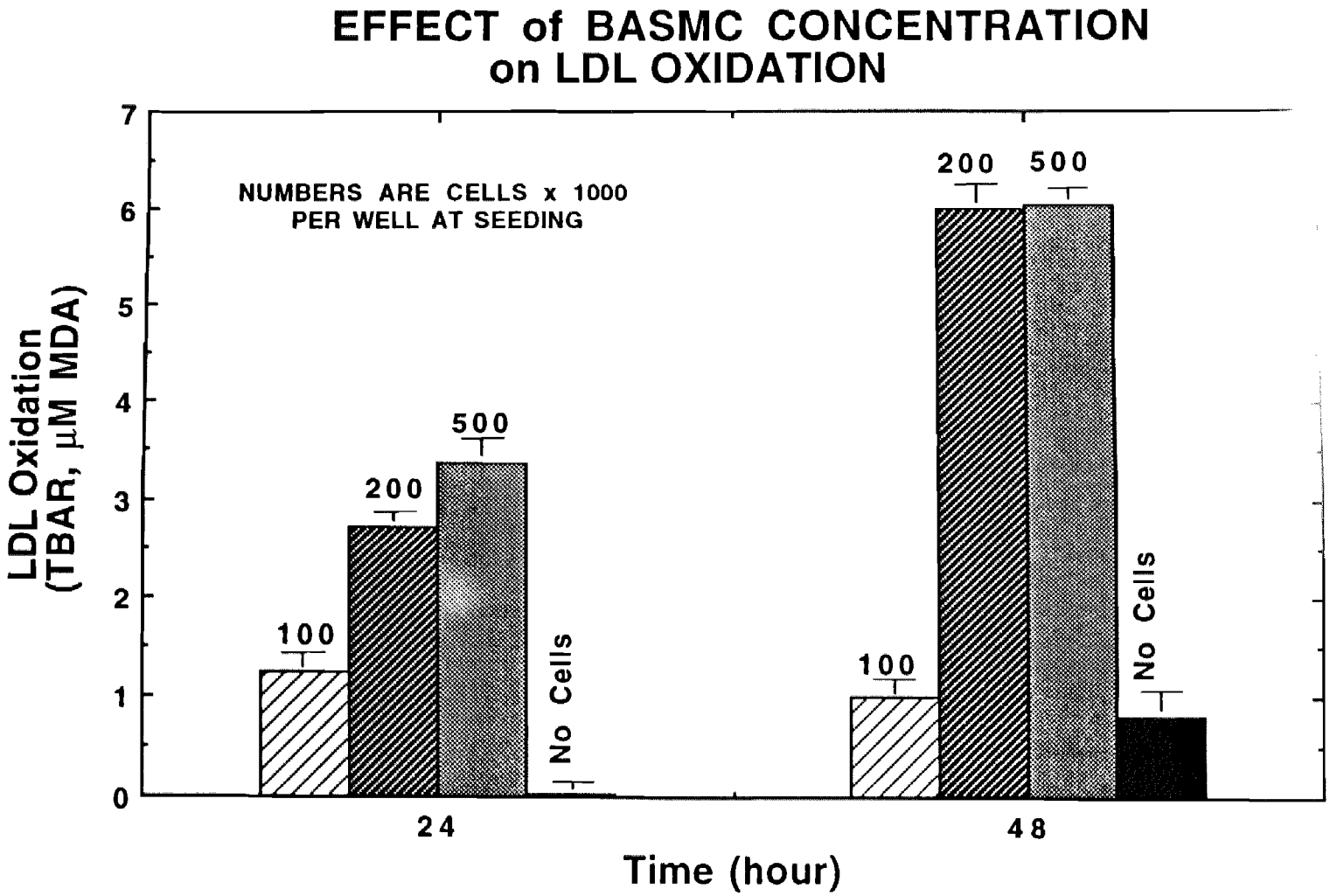
EFFECT of CuSO4 on BASMC OXIDATION of LDL

Treatment	LDL Oxidation (μm MDA/24h)	
BASMC	0.14	(± 0.03)
BASMC + LDL	1.18*	(± 0.26)
BASMC + CuSO4	0.23	(± 0.21)
BASMC + LDL + CuSO4	7.02*	(± 0.28)
LDL	0.69*	(± 0.03)
LDL + CuSO4	2.88*	(± 0.21)

*Significant at $p < 0.05$.

Figure 2. Effect of BASMC concentration on LDL oxidation.

BASMC were seeded on 6-well 9.5 cm^2 plates at the indicated concentrations. Cells were allowed to settle for two days in growth medium. After two days (0 hour), the cells were rinsed with PBS and fed treatment medium. LDL (320 μg/ml) was added to the medium on the cells. At the indicated times, the medium was collected and aliquots analyzed for TBAR material as described in the Methods section. Values represent the mean ± SEM of determinations from triplicate samples.


EFFECT of BASMC CONCENTRATION
on LDL OXIDATION
NUMBERS ARE CELLS x 1000
PER WELL AT SEEDING
LDL Oxidation
(TBAR, μM MDA)
0
1
2
3
4
5
6
7
100
200
500
No Cells
100
200
500
No Cells
24
48
Time (hour)

48 hours, LDL was oxidized to the same extent by cells seeded at either 5 x 10^5 cells/well or 2 x 10^5 cells/well. This rate of oxidation was approximately 5.5 fold that of 1 x 10^5 cells/well, and 7.6 fold that of the auto-oxidation of LDL. In summary, Figure 2 demonstrates that the extent of oxidation of LDL depends on the number of cells present.

Following the demonstration that BASMC could oxidize LDL, the effect of the oxidized LDL on the cells' viability was of interest. Previous work with oxidatively modified LDL indicated that it was cytotoxic to a variety of cells, including aortic smooth muscle cells (2,3). BASMC were seeded on 6-well 9.5 cm^2 plates in growth medium at 5 x 10^5 cells/well, and allowed to grow to confluence. Cells were re-fed treatment medium (0 hour) containing the following: LDL (320 μg/ml), $CuSO_4$ (5 μM), or LDL + $CuSO_4$ (Table 2). After 24 hours, the results showed that the cells remained viable (~ 50% of the 0 hour protein remained) for all treatment groups, excluding the BASMC + LDL. LDL modified by the cells (cell-induced oxidation) proved to be mildly cytotoxic (~ 30% of the 0 hour protein was lost) at the 24 hour time point. These results agree with those published by others using oxidized LDL and bovine aortic smooth muscle cells (2, 3).

Effects of Ketoconazole (KC) on LDL Oxidation

Once the BASMC were characterized (growth and LDL oxidizing abilities), attention was turned to the drug KC. KC is known to bind to the iron in heme compounds and has been shown to inhibit cytochrome P-450 enzymes containing iron (7). KC's ability to lower or prevent the cellular oxidation of LDL, however, is not known.

BASMC were seeded on 6-well 9.5 cm^2 plates in growth medium at 5 x 10^5 cells/well, and allowed to grow to confluence. At confluence, the cells were re-fed treatment medium containing LDL (320 μg/ml), and/or KC (5, 10, or 20 μM). The results in Figure 3 showed that KC lowered the rate of cellular oxidation of LDL in a concentration

Table 2. Effect of LDL and $CuSO_4$ on BASMC viability.

BASMC were seeded on 6-well 9.5 cm^2 plates at a concentration of 5 x 10^5 cells/well. The cells were grown to confluence (4-6 days) in growth medium. At confluency (0 hour), the cells were rinsed with PBS and fed treatment medium. The treatment substances (320 µg LDL/ml, and 5 µM $CuSO_4$) were added directly to the medium on the cells. After 24 hours, samples were collected and analyzed for protein as described in the Methods section. The percent of protein remaining after 24 hours was determined using the mean of determinations from triplicate samples. The data were subjected to the correlated groups t-test, and the differences between BASMC, BASMC + $CuSO_4$, and BASMC + LDL + $CuSO_4$ were found not to be significant ($P < 0.05$).

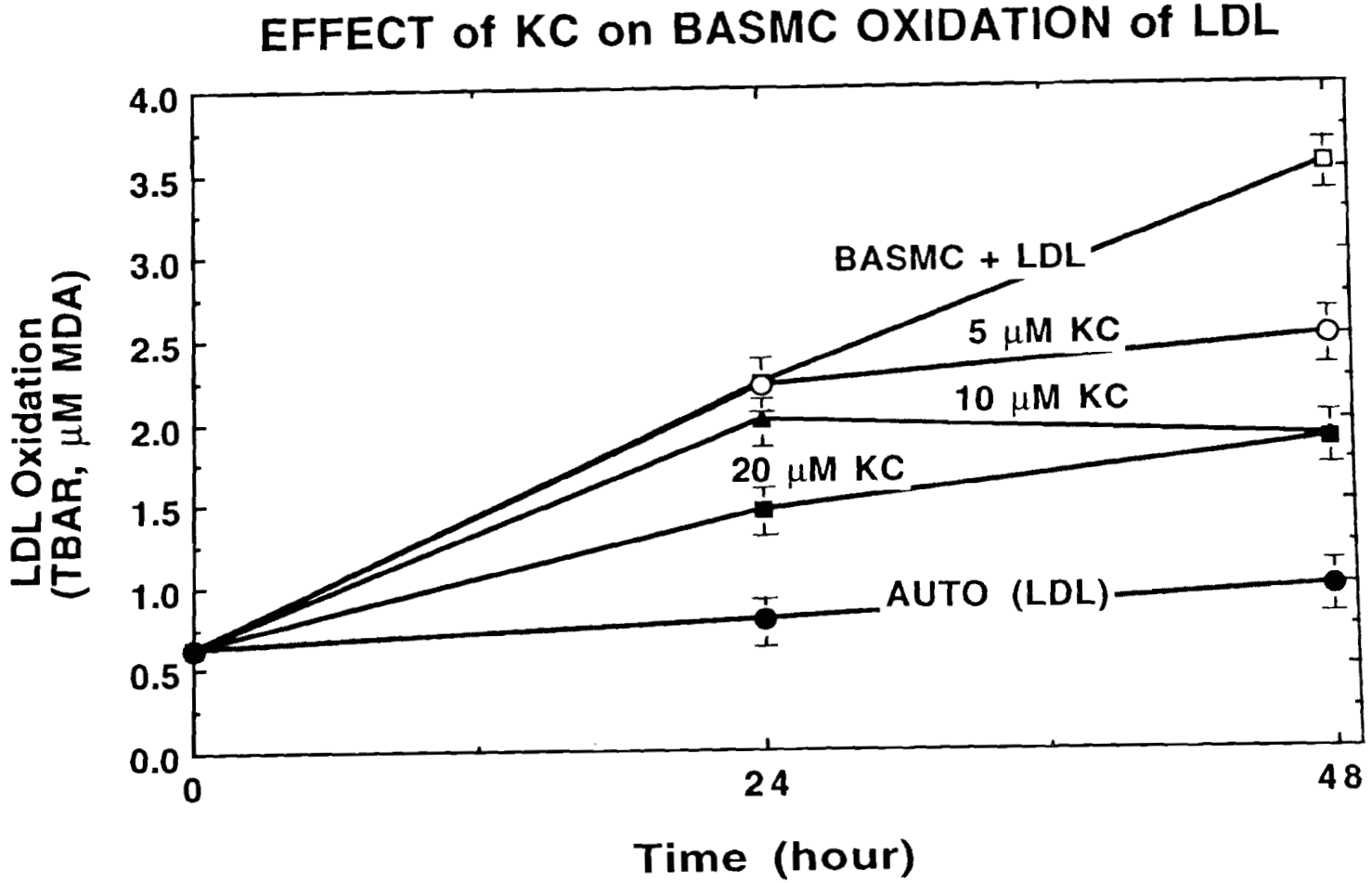
EFFECT of LDL and CuSO4 on BASMC VIABILITY

Treatment	Decrease in Protein/well (% of Control)
BASMC	53%*
BASMC + LDL	72%
BASMC + CuSO4	56%*
BASMC + LDL + CuSO4	52%*

*Not significantly different at $p < 0.05$.

Figure 3. Effect of Ketoconazole (KC) on BASMC oxidation of LDL.

BASMC were seeded on 6-well 9.5 cm^2 plates at a concentration of 5 x 10^5 cells/well. The cells were grown to confluence (4-6 days) in growth medium. At confluency (0 hour), the cells were rinsed with PBS and fed treatment medium. The treatment substances were added at the indicated concentrations to the medium on the cells. At the indicated times, the medium was collected and aliquots analyzed for TBAR material as described in the Methods section. Values represent the mean $\pm$ SEM of determinations from triplicate samples.



10^{-5} At the ls. ial ms

dependent manner. After 48 hours of treatment, 5 μM KC lowered the rate of cellular oxidation of LDL 1.5 fold, while 10 and 20 μM KC lowered the rate approximately 1.8 fold. None of the three KC concentrations were able to lower the rate of LDL oxidation to that of the auto-oxidation of LDL.

In order to show that KC's inhibition of cellular oxidation of LDL was not due to cell toxicity, we were interested in determining the effects of KC on cell viability. BASMC were seeded on 6-well 9.5 cm^2 plates in growth medium at 1, 3, and 5 x 10^5 cells/well (Figure 4). The cells were allowed to settle. At 0 hour, the cells were re-fed treatment medium containing KC (5, 10, or 20 μM). Control cells (0 μM KC) were fed an equal amount of EtOH to ensure results were not the product of EtOH toxicity. The results shown in Figure 4 indicate that KC, regardless of concentration, was not cytotoxic. The loss in cellular protein is comparable to the loss seen with the control cells, and might be due to the lack of nutrients in the treatment medium.

Now that it was known that KC in the treatment medium lowered the rate of LDL oxidation and was not cytotoxic, we were interested in determining if KC could lower the rate of LDL oxidation so that the LDL became less cytotoxic. BASMC were seeded on 6-well 9.5 cm^2 plates in growth medium at 5 x 10^5 cells/well, and allowed to grow to confluence (Table 3). At 0 hour, the cells were re-fed treatment containing LDL (320 μg/ml) and the indicated KC concentration. After 24 hours, KC was found to prevent the loss of cellular protein in a concentration dependent manner (20 μM KC protected better than 10 μM, etc.) when compared to the BASMC and BASMC + LDL controls. Approximately 66% of the cellular protein remained after 24 hours for the BASMC, BASMC + LDL, and BASMC + LDL + 20 μM KC, indicating that the KC and LDL were not cytotoxic. After 48 hours, the BASMC + LDL + 20 μM KC showed increased retention of cellular protein (65%) when compared to the BASMC (51%) and BASMC +

Figure 4. Effect of Ketoconazole (KC) concentration on BASMC' viability in treatment medium.

BASMC were seeded on 6-well 9.5 cm^2 plates at the indicated concentrations. Cells were allowed to settle for two days in growth medium. After two days (0 hour), the cells were rinsed with PBS and fed treatment medium. KC was added to the medium on the cells at the indicated concentrations. At the indicated times, samples were collected and analyzed for protein as described in the Methods section. Values represent the mean $\pm$ SEM of determinations from triplicate samples.

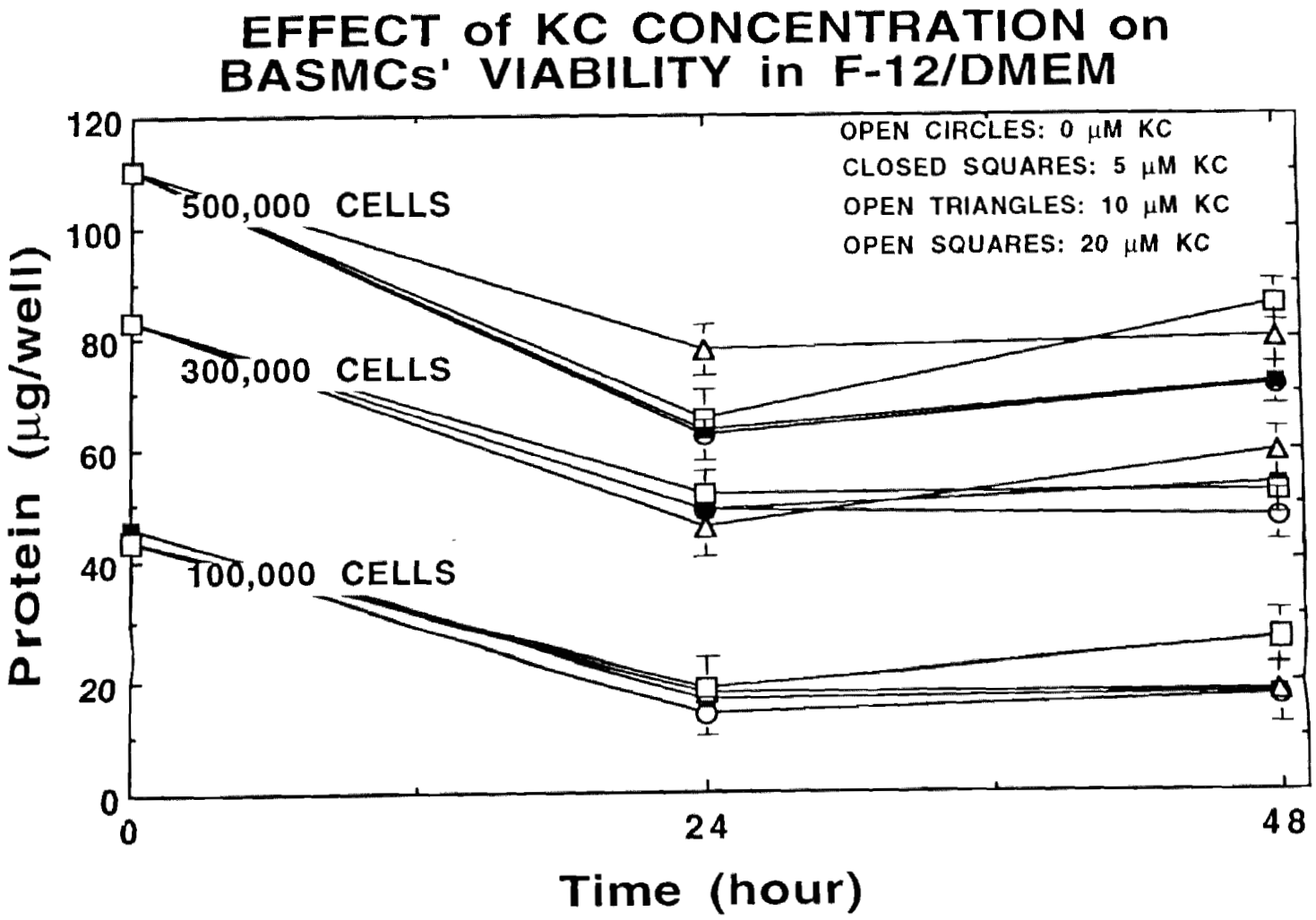

EFFECT of KC CONCENTRATION on
BASMCs' VIABILITY in F-12/DMEM
OPEN CIRCLES: 0 μM KC
CLOSED SQUARES: 5 μM KC
OPEN TRIANGLES: 10 μM KC
OPEN SQUARES: 20 μM KC
500,000 CELLS
300,000 CELLS
100,000 CELLS
Protein (μg/well)
120
100
80
60
40
20
0
0
24
48
Time (hour)

Table 3. Effect of Ketoconazole (KC) and LDL on BASMC viability.

BASMC were seeded on 6-well 9.5 cm^2 plates at a concentration of 5 x 10^5 cells/well. The cells were grown to confluence (4-6 days) in growth medium. At confluency (0 hour), the cells were rinsed with PBS and fed treatment medium. The treatment substances (KC at the indicated concentrations, and 320 µg LDL/ml) were added to the medium on the cells. At the indicated times, samples were collected and cell viability determined as described in the Methods section. Values represent the mean of determinations from triplicate samples. SEM is given in parentheses.

EFFECT of KC & LDL on BASMC VIABILITY

Treatment	Decrease in Cell number/well (% of Control) 24 hour	48 hour
BASMC	65% (±1.2%)	51% (±3.3%)
BASMC + LDL	67% (±2.1%)	49% (±2.4%)
BASMC + LDL + 5 uM KC	48% (±1.3%)	50% (±2.7%)
BASMC + LDL + 10 uM KC	55% (±2.1%)	52% (±4.9%)
BASMC + LDL + 20 uM KC	66% (±0.3%)	65% (±2.1%)
BASMC + 5 uM KC	81% (±1.5%)	63% (±3.9%)
BASMC + 10 uM KC	73% (±3.6%)	70% (±5.5%)
BASMC + 20 uM KC	67% (±0.2%)	61% (±1.0%)

LDL (49%) controls, indicating that the KC in combination with LDL had a protective effect.

Electrophoresis of Oxidized LDL

Agarose gel electrophoresis was performed to give a visual representation of the extent of LDL oxidation (Figure 5 and Table 4). BASMC were seeded on 6-well 9.5 cm^2 plates in growth medium at 5 x 10^5 cells/well, and allowed to grow to confluence. On day 0, the BASMC were re-fed treatment medium containing LDL (320 µg/ml) plus or minus treatment substances. After 24 and 48 hours, LDL samples from the medium were collected and subjected to electrophoresis. The agarose gel electrophoresis data yielded the following results: 1) LDL oxidation (not TBAR formation) was shown by the migration of the bands in the gels (Figure 5), and the change in the REM values (Table 4). LDL modified by BASMC moved further through the gel than LDL that was auto-oxidized, and cell-oxidized LDL had greater REM values (1.27 at 48 hours) than LDL that was auto-oxidized (1.01 at 48 hours). 2) KC's prevention of LDL oxidation was supported by the decrease in REM values, or the lack of change from zero hour control (Table 4). At 48 hours, the REM value for LDL incubated with BASMC and 20 µM KC was lower (1.13) than that of LDL incubated with BASMC (1.27). Overall, the electrophoresis data supports the TBAR evidence that oxidation took place, and that KC lowered cellular oxidation of LDL.

Cell Photography of BASMC after LDL and KC Treatment

Visual inspection of BASMC was used to ascertain the morphological effects of the various treatments on the cells. BASMC were seeded on 6-well 9.5 cm^2 plates, or tissue culture slides, in growth medium at 5 x 10^5 cells/well, and allowed to grow to confluence (Figure 6). At confluency, the cells were re-fed treatment medium containing the treatment substances. All photos were taken after 24 hours of treatment. The results are given

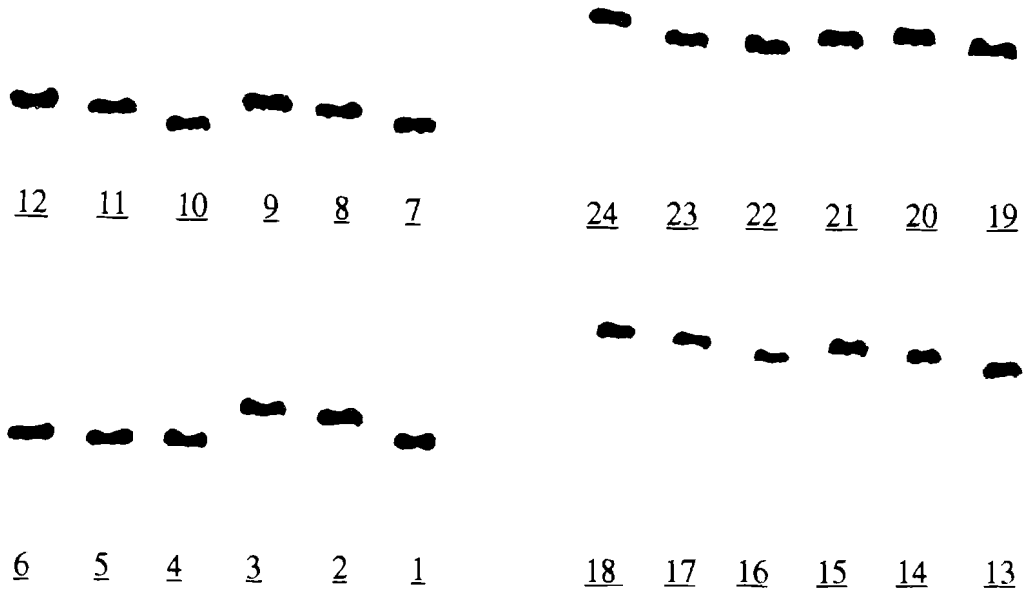


Figure 5. Agarose gel electrophoresis of LDL after treatment.

BASMC were seeded in growth medium on 6-well 9.5 cm^2 plates at a concentration of 5 x 10^5 cells/well, and allowed to grow to confluence. On day 0, the BASMC were fed treatment medium containing LDL (320 µg/ml) plus or minus treatment substances. At the indicated times, aliquots of medium were collected and subjected to Agarose gel electrophoresis (5.1 µg LDL/lane) as described in the Methods section. Lanes contain LDL treated as follows:

0 h	**24 h**	**48 h**
1 BASMC	2 BASMC	3 BASMC
4 LDL (auto-ox.)	5 LDL (auto-ox.)	6 LDL (auto-ox.)
7 BASMC + 10 µM KC	8 BASMC + 10 µM KC	9 BASMC + 10 µM KC
10 BASMC + 5 µM KC	11 BASMC + 5 µM KC	12 BASMC + 5 µM KC
13 BASMC + 20 µM KC	14 BASMC + 20 µM KC	15 BASMC + 20 µM KC
16 5 µM KC	17 5 µM KC	18 5 µM KC
19 10 µM KC	20 10 µM KC	21 10 µM KC
22 20 µM KC	23 20 µM KC	24 20 µM KC

Table 4. Relative electrophoretic mobility values of LDL after treatment.

The relative electrophoretic mobility (REM) values from Figure 5 are presented in Table 4. REM values were determined by measuring the distance (in millimeters) between the center of the well and the center of the LDL band. For each treatment group, the zero hour value served as the control.

RELATIVE ELECTROPHORETIC MOBILITY VALUES of LDL after TREATMENT

LANE	TREATMENT	HOUR	REM
1	BASMC	0	1.00
2	BASMC	24	1.20
3	BASMC	48	1.27
4	auto-ox.	0	1.00
5	auto-ox.	24	1.00
6	auto-ox.	48	1.01
7	BASMC+10 μM KC	0	1.00
8	BASMC+10 μM KC	24	1.22
9	BASMC+10 μM KC	48	1.27
10	BASMC+5 μM KC	0	1.00
11	BASMC+5 μM KC	24	1.24
12	BASMC+5 μM KC	48	1.29
13	BASMC+20 μM KC	0	1.00
14	BASMC+20 μM KC	24	1.06
15	BASMC+20 μM KC	48	1.13
16	5 μM KC	0	1.00
17	5 μM KC	24	1.12
18	5 μM KC	48	1.18
19	10 μM KC	0	1.00
20	10 μM KC	24	1.20
21	10 μM KC	48	1.18
22	20 μM KC	0	1.00
23	20 μM KC	24	1.04

Light Microscopy. Magnification of 100 x. A. Control (growth medium) versus B. Control (treatment medium). Slight effect of serum deprivation noted after 24 hours. Cell density for cells in treatment medium was not as high as cells in growth medium. There were gaps between the cells in treatment medium (they were not in as tight of contact as the cells in growth medium).

A. Control (growth medium) versus C. LDL. Loss of cell density noted due to treatment with LDL.

A. Control (growth medium) versus D. 20 μM KC. Only slight loss of cell density (comparable to the loss due to treatment medium) noted due to treatment with KC.

A. Control (growth medium) versus E. LDL + 20 μM KC. Only slight loss of cell density (comparable to the loss due to treatment medium) noted due to treatment with LDL + KC.

A. Control (growth medium) versus F. LDL + $CuSO_4$. Irritating effect and loss of cell density noted due to LDL + $CuSO_4$ treatment. The cells' appeared to be rounding up, and they were no longer in tight contact with one another.

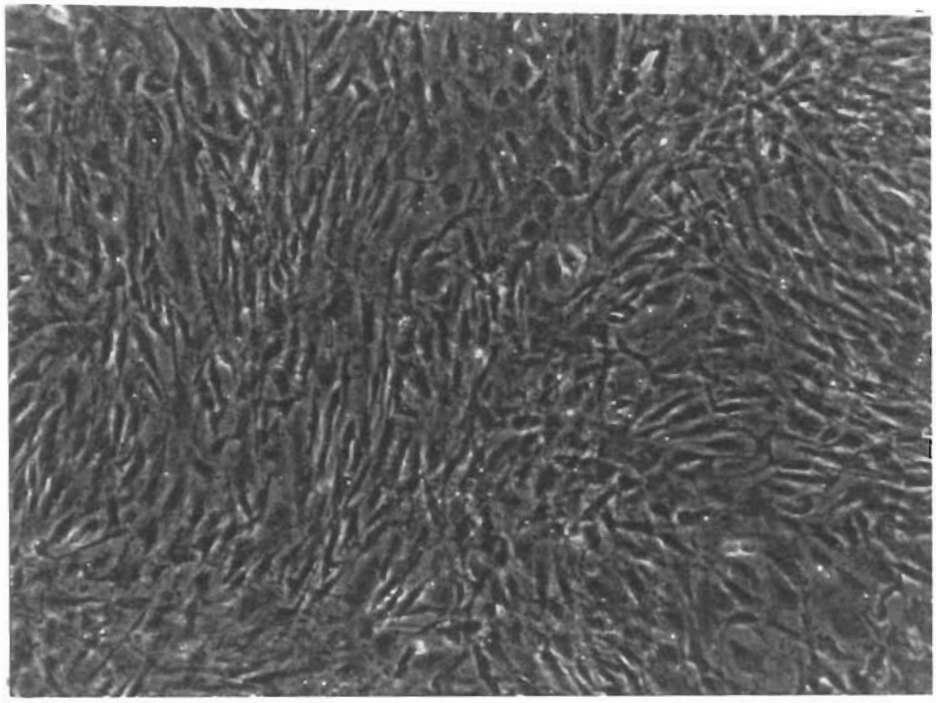
Electron Microscopy. Magnification of 1680 x. G. Control (growth medium) versus H. Control (treatment medium). No apparent effect of treatment noted. Cells' surfaces appeared to be smooth, there were tight contacts between cells, and the ends of cells were not frayed.

G. Control (growth medium) versus I. LDL. LDL's detrimental effects on cells noted. Cells' surfaces were not as smooth as control cell surfaces. Cells were retracting from each other (no longer in tight contact), and the ends of the cells were frayed. Cell detritus was also noted.

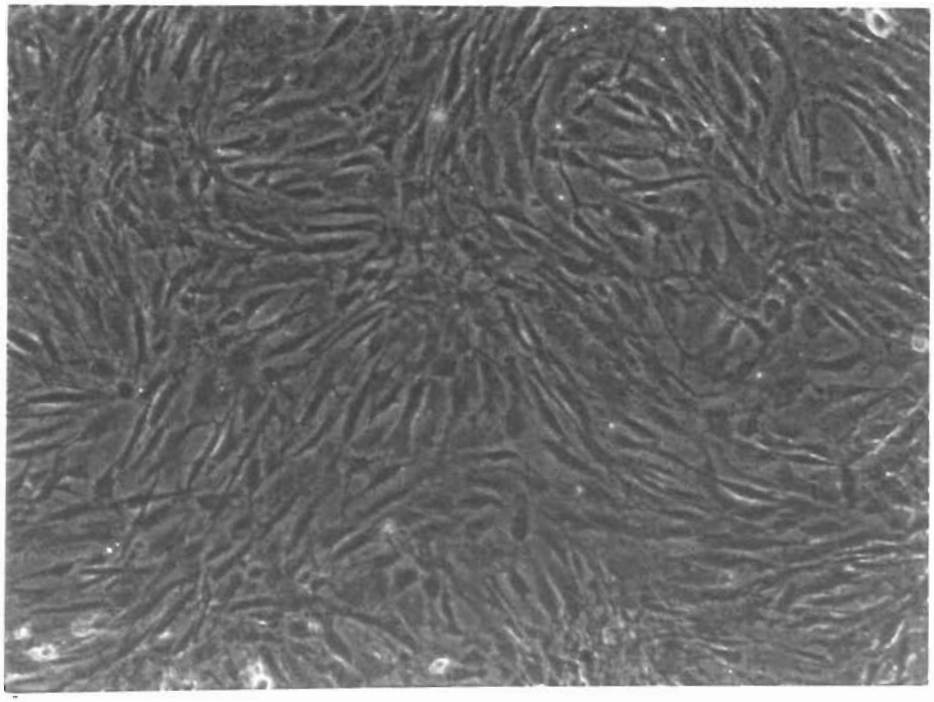
G. Control (growth medium) versus J. 20 μM KC. Slight irritating effect of KC on cells was observed. Cells' surfaces were not as smooth, but there were tight contacts

Figure 6. Photos (A - V) of BASMC after treatments.

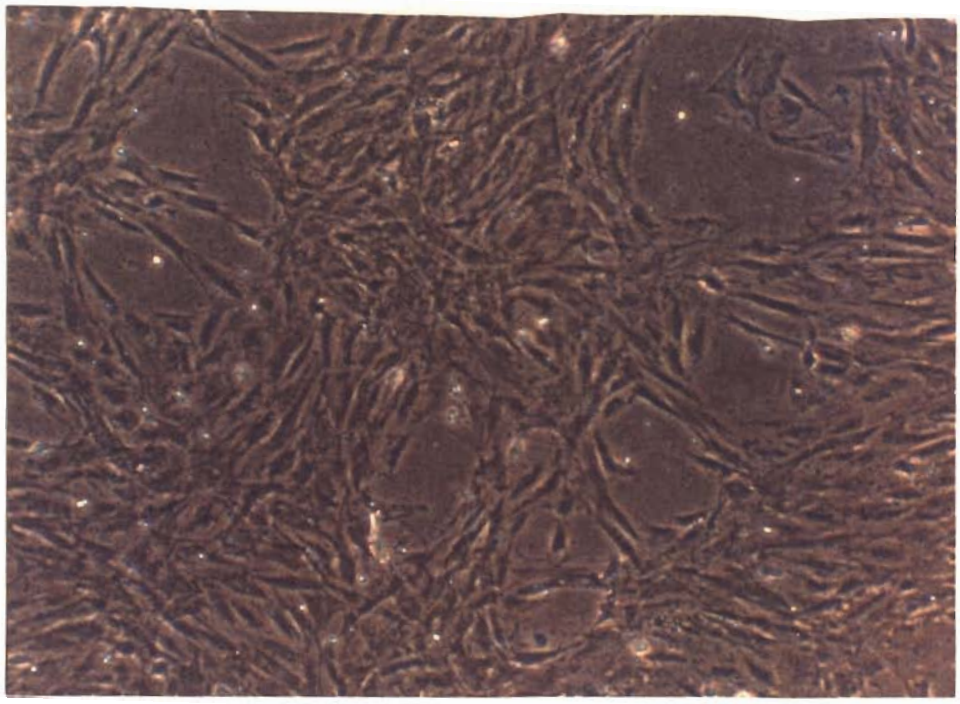
BASMC were seeded on 6-well 9.5 cm^2 plates, or tissue culture slides, at a concentration of 5 x 10^5 cells/well. The cells were grown to confluence (4-6 days) in growth medium. At confluency (0 hour), the cells were rinsed with PBS and fed treatment medium. The treatment substances, as indicated, were added directly to the medium on the cells. At the indicated times, light microscopy pictures were taken, or cells were collected and prepared for electron microscopy as described in the Methods section. Legend: **D** indicates cell detritus, and **F** indicates frayed edges of cells.



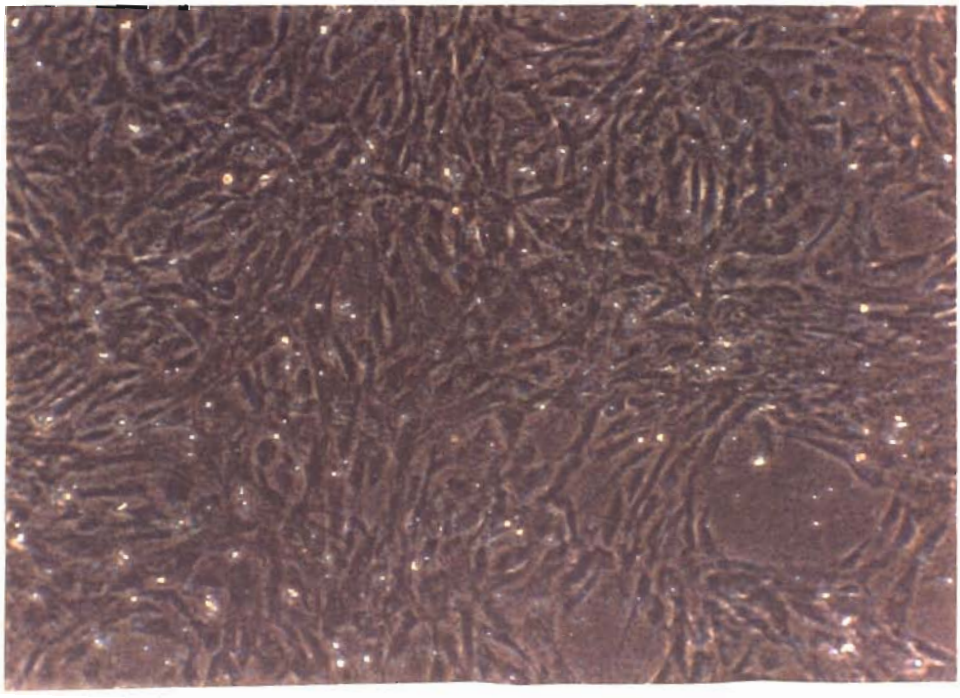
A. Control (growth medium), 24 hours. 100 x.

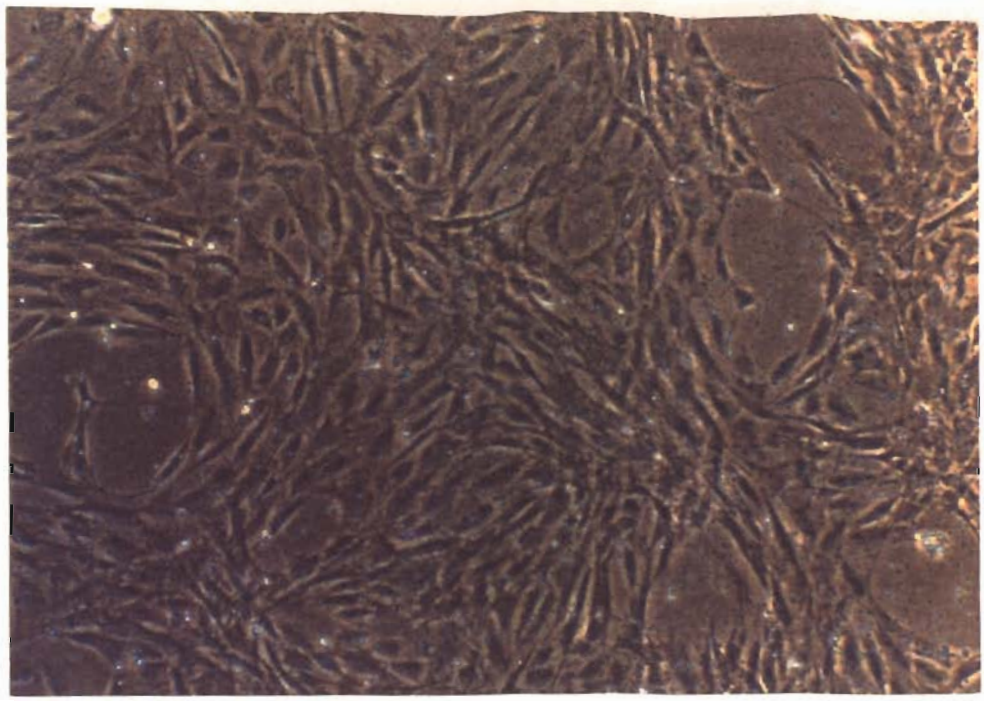


B. Control (treatment medium), 24 hours. 100 x.

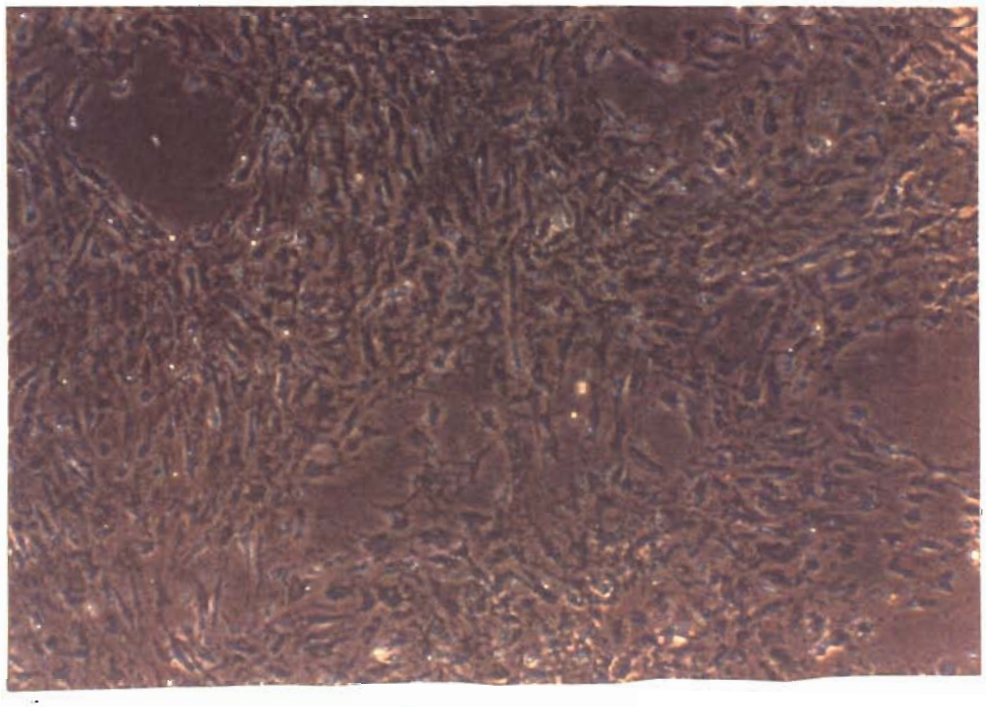


C. LDL, 24 hours. 100 x.



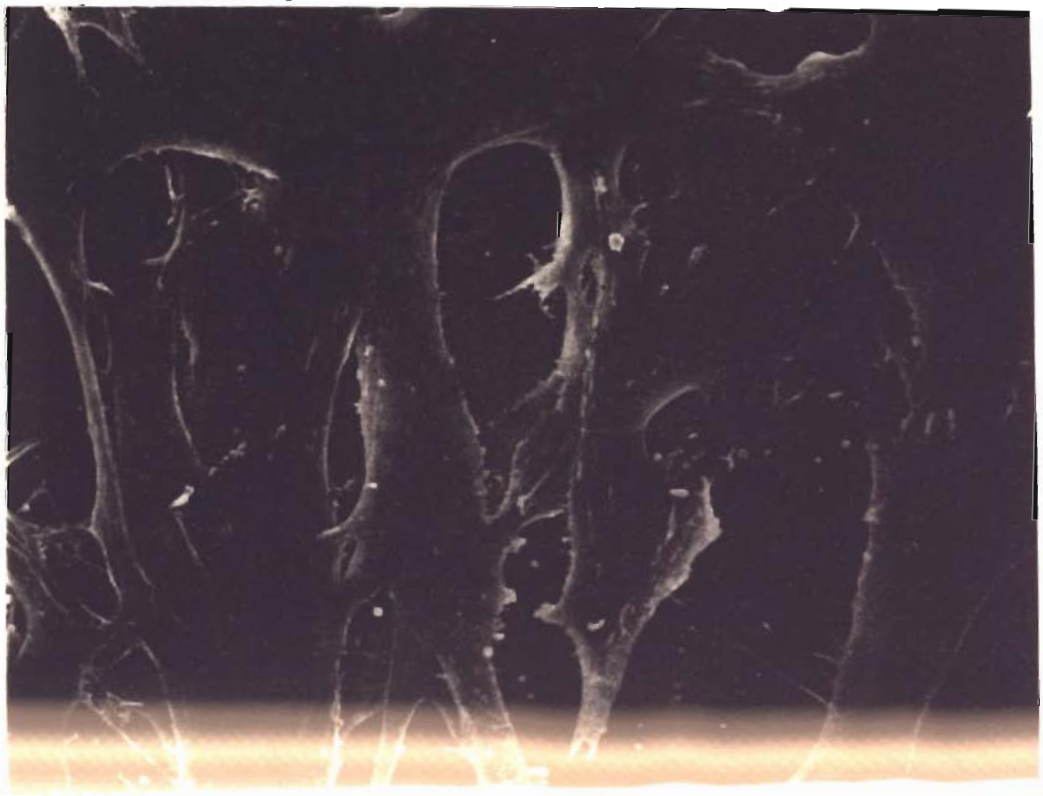


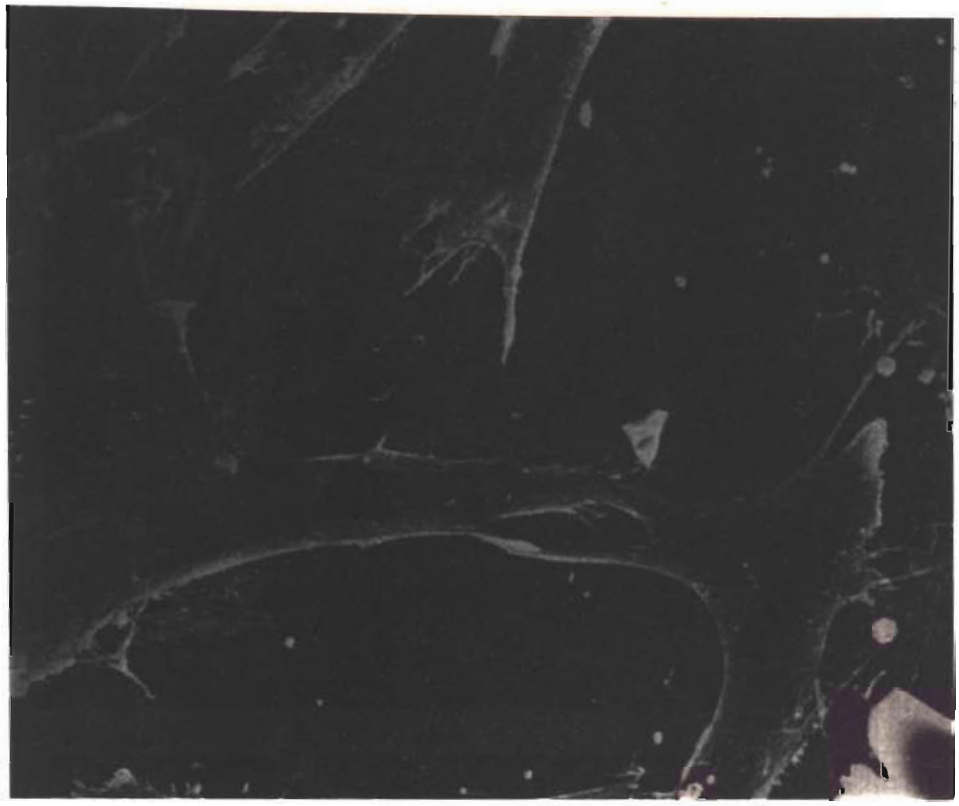
E. LDL + 20 μM KC, 24 hours. 100 x.



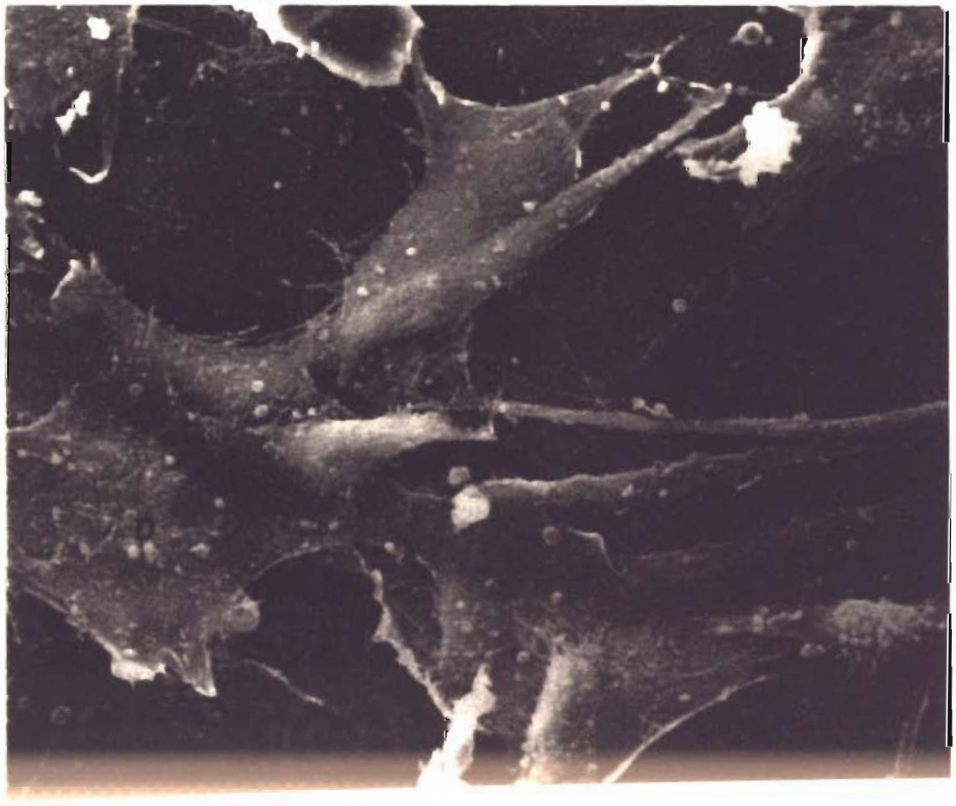
F. LDL + $CuSO_4$, 24 hours. 100 x.

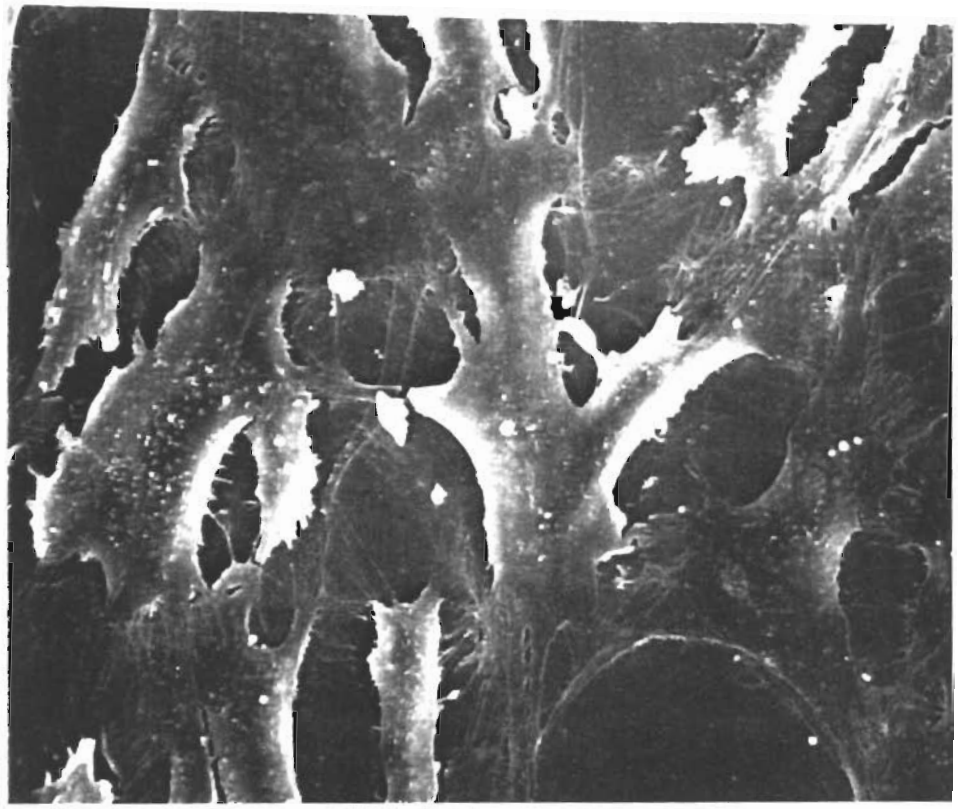
G. Control (growth medium), 24 hours. 1680 x.



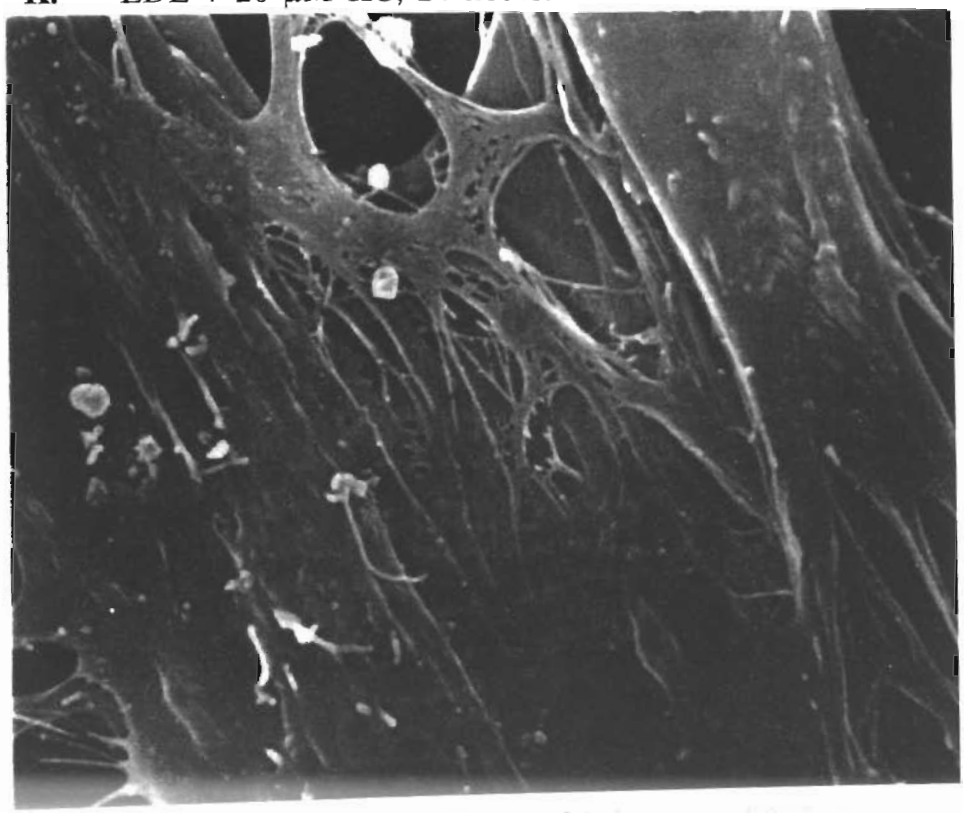


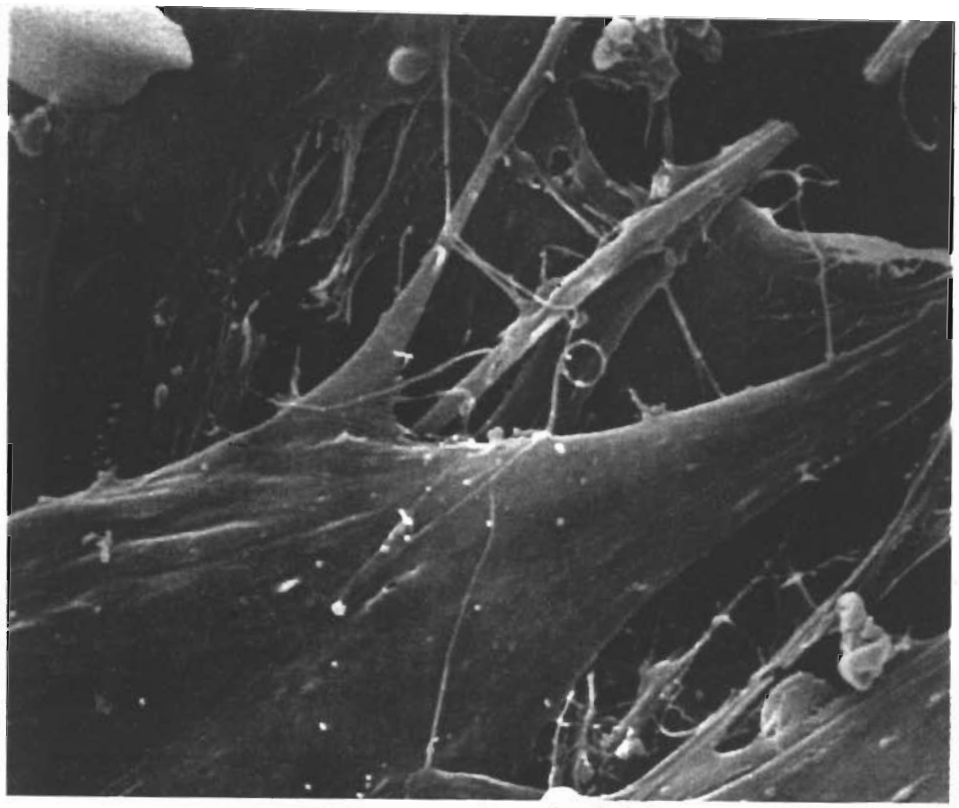
I. LDL, 24 hours. 1680 x.



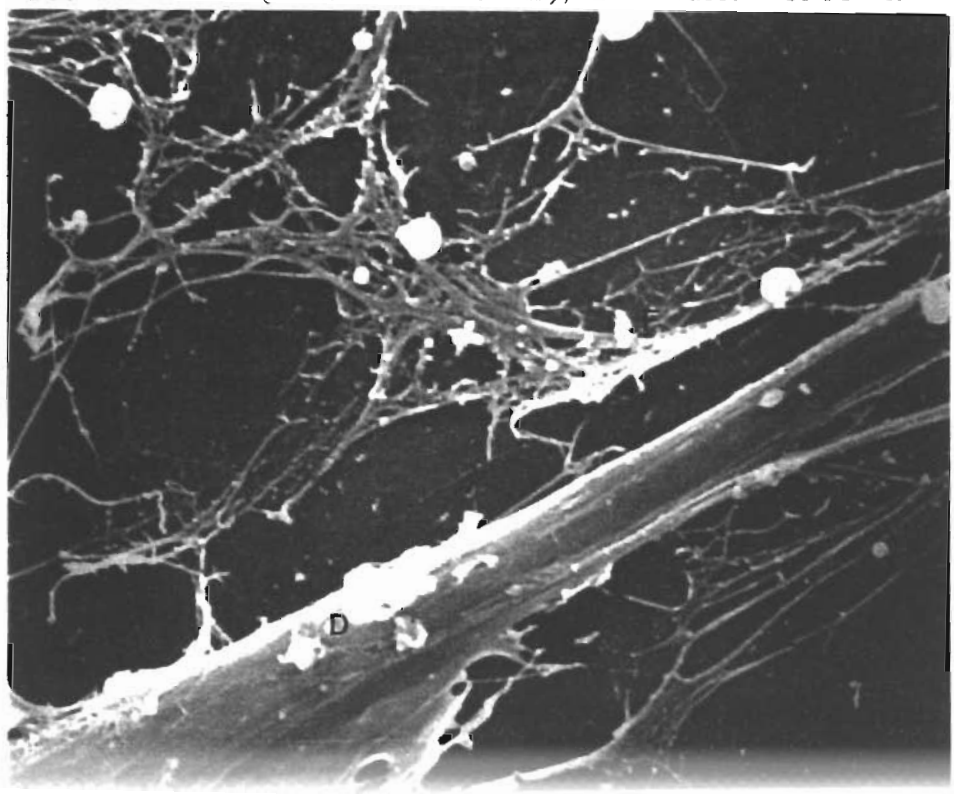


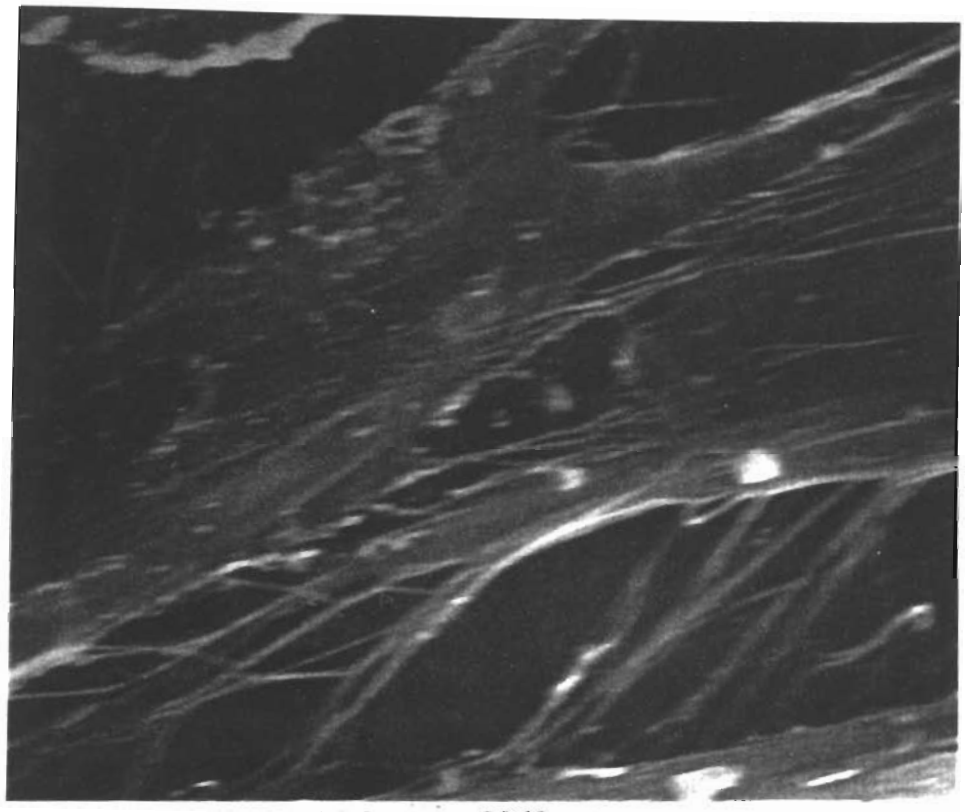
K. LDL + 20 μM KC, 24 hours. 1680 x.



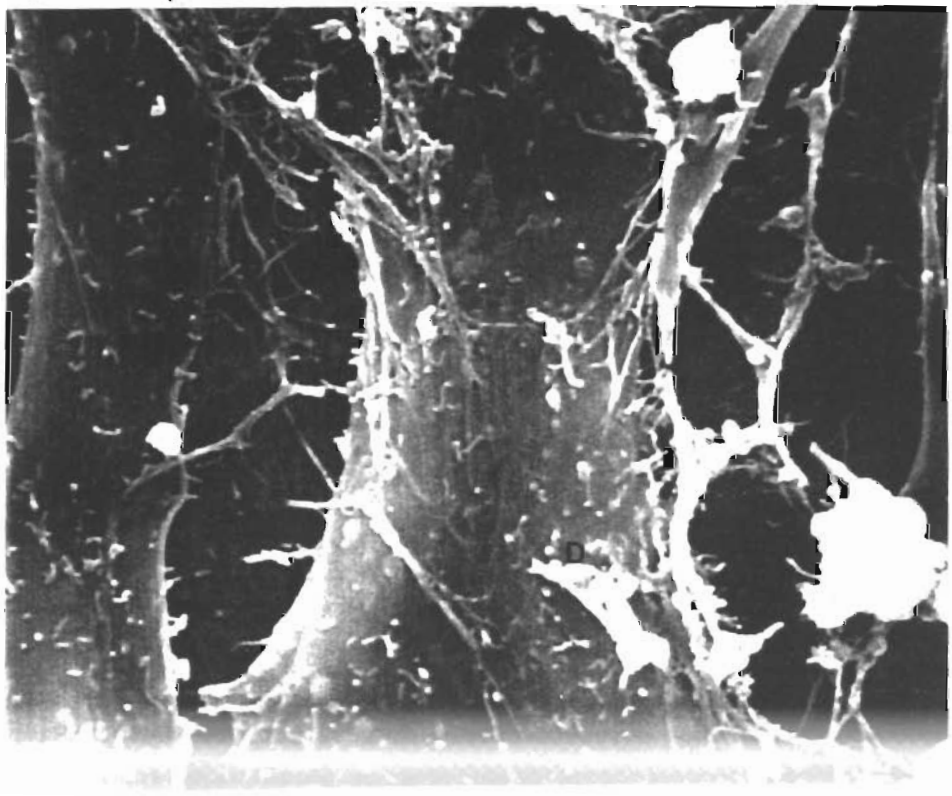


M. Control (treatment medium), 24 hours. 8960 x.





O. 20 μM KC, 24 hours. 8960 x.



G. Control (growth medium) versus K. LDL + 20 μM KC. No apparent effect of LDL and KC treatment on cells was noted. Cells' surfaces appeared smooth (they resembled control cells' surfaces). There were tight contacts between cells, and the contact points between cells were not frayed.

Magnification of 8960 x. L. Control (growth medium) versus M. Control (treatment medium). No apparent effect of treatment noted. Cells' surfaces appeared smooth, and there were tight contacts between cells.

L. Control (growth medium) versus N. LDL. LDL's detrimental effects on cells were seen. Cells' surfaces did not appear as smooth, and the ends of cells were stringy and frayed (due to the cells pulling away from each other). Detritus was observed to be clinging to the cells.

L. Control (growth medium) versus O. 20 μM KC. The slight effect of KC treatment on cells was noted. Cells' surfaces were not as smooth, and there appeared to be a slight pulling away between the cells.

L. Control (growth medium) versus P. LDL + 20 μM KC. No apparent effect of LDL and KC treatment on cells was seen. Cells' surfaces appeared smooth, and resembled control cells' surfaces. There were tight contacts between cells, and the ends of cells were not frayed. There was some cell detritus present.

Effects of Celox on BASMC Growth and Oxidation of LDL

Protein analysis of the BASMC after the various treatments (LDL, KC, etc.) revealed that approximately fifty percent of the total zero hour protein for the cells was being lost by the 24 hour time point. It was hypothesized that the cells were suffering from the lack of fetal calf serum (FCS), a component essential for their growth and survival (14), which was not added to the treatment medium (FCS interferes with the BASMC oxidation of LDL, therefore it was not added to the medium used in LDL oxidation experiments). Celox, newly marketed as a serum replacement, may not interfere with

BASMC oxidation of LDL, and yet maintain cell viability. We therefore determined if Celox would improve the survival rate for the BASMC (if it would prevent the cells from dying from serum starvation), and if the BASMC could oxidize LDL in the treatment medium with Celox.

The effect of Celox on BASMC, as measured by cellular protein adhering to the well, is shown in Table 5. After 48 hours, BASMC with Celox had a protein retention of 50% of the zero hour protein value, compared to 22% for BASMC in treatment medium alone. After 96 hours, 58% of the zero hour protein remained for the BASMC with Celox, as opposed to 8% for the BASMC in the treatment medium alone. When LDL was added, 50% of the zero hour protein remained after 48 hours for the BASMC in the medium with Celox, while only 35% remained for the cells without Celox. After 96 hours with LDL, 35% of the zero hour protein remained for the BASMC with Celox, while only 25% remained for the cells without Celox. The addition of the supplement Celox to the treatment medium improved the BASMCs' viability by as much as 50% (as indicated by the protein assay) when compared to the cells' viability in the treatment medium without Celox.

Now that it was known that the addition of Celox to the medium improved cell viability, it needed to be determined if the BASMC could oxidize LDL in medium containing Celox. It was found that the BASMC incubated with LDL in both the treatment medium and the treatment medium containing Celox were able to oxidize the LDL (Figure 7.). After 96 hours, the LDL + BASMC incubated in the treatment medium had a TBAR value of 4.3, while the LDL + BASMC + Celox in treatment medium had a TBAR value of 6.3. The 1.5 fold increase in oxidation seen with the LDL incubated with Celox could have been due to the higher number of viable cells present (the more cells, the higher the rate of oxidation) due to the addition of the Celox. Although the addition of Celox to the medium caused a slight increase in the auto-oxidation, statistically, the difference was not significant (correlated groups t-test, $P < 0.05$). The results of the TBAR assay indicated that Celox

Table 5. Effect of Celox and LDL on BASMC viability.

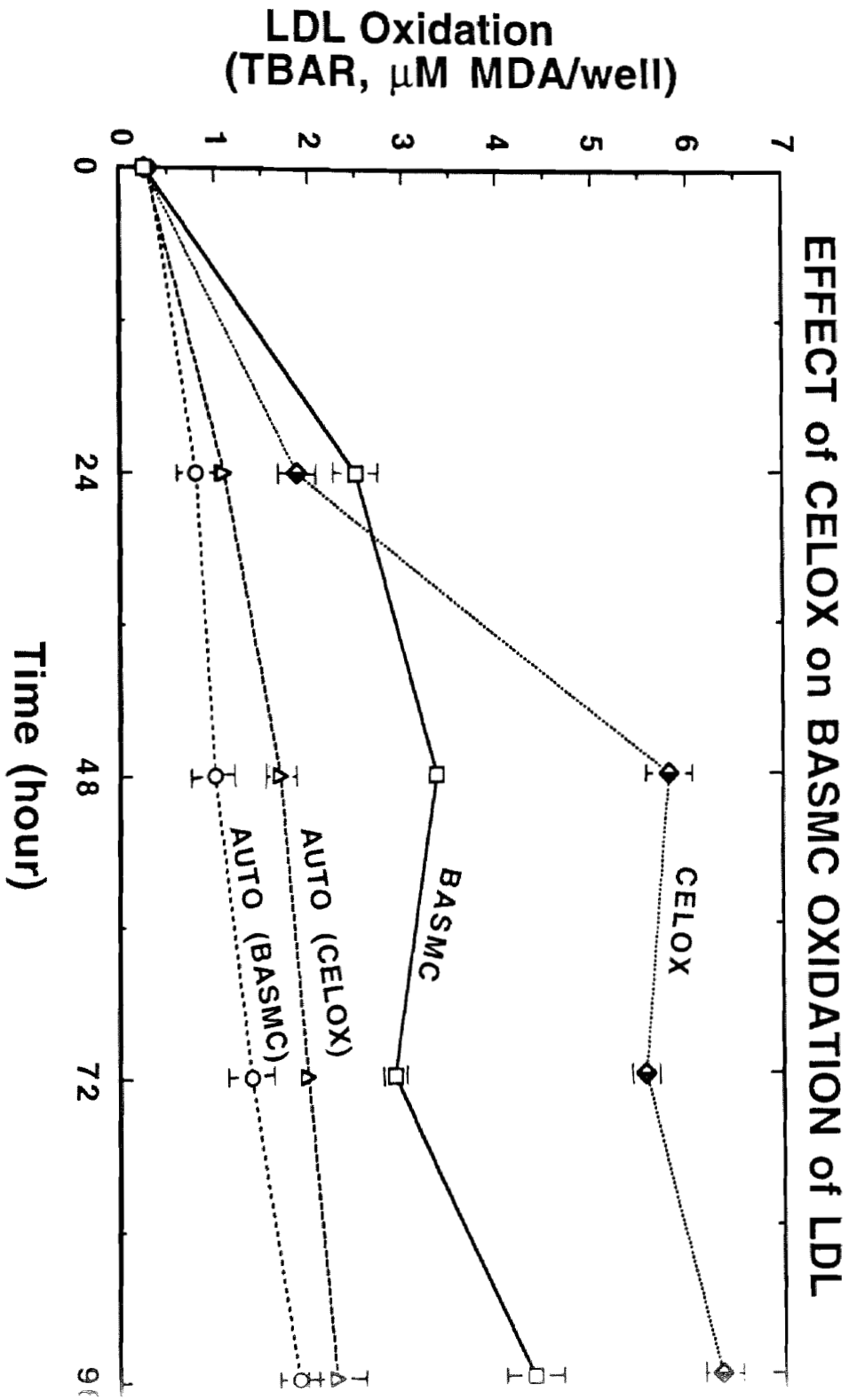
BASMC were seeded on 24-well 2 cm^2 plates at a concentration of 1 x 10^5 cells/well. The cells were grown to confluence (4-6 days) in growth medium. At confluency (0 hour), the cells were rinsed with PBS and fed treatment medium. The treatment substances (Celox and 320 µg LDL/ml) were added to the medium on the cells. At the indicated times, samples were collected and analyzed for protein as described in the Methods section. Values represent the mean of determinations from triplicate samples. SEM is given in parentheses.

EFFECT of CELOX & LDL on BASMC VIABILITY

Treatment	Decrease in Protein/well (% of Control) Day 1	Day 2	Day 3	Day 4
TREATMENT MEDIA	37% (±1%)	22% (±0%)	17% (±4%)	8% (±2%)
CELOX	46% (±2%)	50% (±0%)	47% (±4%)	58% (±10%)
TREATMENT MEDIA + LDL	42% (±4%)	35% (±2%)	29% (±3%)	25% (±4%)
CELOX + LDL	40% (±9%)	50% (±2%)	31% (±4%)	35% (±3%)

Figure 7. Effect of Celox on BASMC oxidation of LDL.

BASMC were seeded on 24-well 2 cm^2 plates at a concentration of 1 x 10^5 cells/well. The cells were grown to confluence (4-6 days) in growth medium. At confluency (0 hour), the cells were rinsed with PBS and fed treatment medium. The treatment substances (Celox and 320 μg LDL/ml) were added to the medium on the cells. At the indicated times, the medium was collected and aliquots analyzed for TBAR material as described in the Methods section. Values represent the mean of determinations from triplicate samples. SEM is given in parentheses.


EFFECT of CELOX on BASMC OXIDATION of LDL
LDL Oxidation
(TBAR, μM MDA/well)
0
1
2
3
4
5
6
7
0
24
48
72
96
Time (hour)
CELOX
BASMC
AUTO (CELOX)
AUTO (BASMC)

did not interfere with the BASMC oxidation of LDL, and given its beneficial effects on cell viability, the addition of Celox to the treatment medium in future experiments was indicated.

DISCUSSION

BASMC Growth Determiners

The BASMC line that was used had a growth pattern that was in accordance with established patterns of growth for monolayer cultures (14). After seeding for an experiment, the cells underwent a quiescent period (lag phase) during which there was no cell division. The length of the lag phase was dependent on several factors, including cell type, seeding density, and medium composition. The cells then entered a log phase of growth, where there was an exponential increase in cell number. The log phase was where the cells exhibited their greatest metabolic activity. When culture conditions reached a state that was no longer ideal for cell division (for example, the cells reached confluence, or the medium was depleted of nutrients), the cell population entered a stationary phase, during which the cells no longer divided and the cell number remained constant .

Growth experiments with BASMC showed that the higher seeding densities (4 and 5 x 10^5 cells/well, Figure 1) reached confluence ("confluence" describes a cell population that occupies all available growth space) first, and that the cells looked healthier under the microscope. There are several explanations for these findings. First, large populations of cells are able to multiple under conditions that smaller populations of cells are unable to multiply under (14). In order for cells to optimally grow and divide, the medium the cells are in needs to be "conditioned". Large populations of cells are able to "condition" the medium faster than small populations. Several factors are involved in conditioning the medium: 1) Inhibitory materials that bind to the cells are neutralized before they reach a level per cell that is high enough to block growth. 2) Low-molecular-weight metabolic intermediates that diffuse out of the cells accumulate in the medium in amounts that maintain adequate intracellular levels for biosynthesis and metabolism. 3) Macromolecules

synthesized by the cells build up to levels needed for growth at a higher rate with larger populations of cells (14).

For all experiments, the BASMC were re-fed medium (treatment medium) that did not contain FCS (the medium the cells were grown in, growth medium, contained FCS). It was observed that after as little as 24 hours in the treatment medium, the BASMC were losing up to 50% of their zero hour protein content. It was hypothesized that the BASMC were dying due to serum starvation (the lack of FCS in the treatment medium). Serum provides many of the raw materials cells need to reproduce new cells, substrates for energy metabolism, and vitamins and trace minerals needed to catalyze reactions (14). Serum is also a major source of macromolecular growth factors that are essential for the multiplication of many cell types, including the BASMC used in this study. Serum is so effective at supporting the multiplication of cells because it contains a large number of different growth factors in a physiologically balanced blend. Serum macromolecules also buffer toxic nutrients by binding them and releasing them in small amounts as their free concentration in the medium is reduced by cellular metabolism.

A majority of the experiments performed in this study, however, had to be carried out in medium without FCS because of serum's protective effect on BASMC. In the presence of FCS, the BASMC would not oxidize LDL to the extent needed for toxicity studies. However, in several experiments, a serum replacement product, Celox, was available. BASMC viability and LDL oxidation experiments were repeated using treatment medium containing Celox to determine what effect Celox would have on the experimental conditions. The BASMC had increased viability in the treatment medium containing Celox (Table 4), and were able to oxidize LDL in Celox's presence (Figure 5). It is, therefore, recommended that any further work done with these BASMC be done in medium containing Celox.

Oxidation of LDL by BASMC

In agreement with earlier reports (2, 5, 6), the BASMC used in these experiments were able to modify LDL. The literature indicates that the changes in LDL produced by incubation with BASMC included greater anodic electrophoretic mobility, greater degradation of the LDL by macrophages, more TBAR material, and a reduced ratio of total cholesterol to protein (8). The results of this study with BASMC support the statement that these cells can oxidize LDL based on the following observations: 1) There was an increase in electrophoretic mobility; 2) There was an increase in TBAR material present; and 3) There was a loss in cell viability.

The oxidation of LDL in the absence of cells (auto-oxidation) was used as a positive control to ensure that the LDL used was capable of being modified. LDL was incubated in treatment medium alone, and in treatment medium containing $CuSO_4$ (5 μM) to establish lower and upper oxidation limits for the LDL (Table 1). The BASMCs' rate of oxidation fell in-between the lower auto-oxidation (treatment medium) and upper auto-oxidation (treatment medium + $CuSO_4$) values, establishing TBAR assay parameters.

LDL was modified without cells being present, but to a much lesser extent than LDL in the presence of cells (Table 1). The presence of trace metal impurities in the treatment medium, such as Cu^{2+}, was a determining factor in the rate of oxidation (6). The LDL particle contains trace amounts of hydroperoxides which are decomposed by the Cu^{2+} to release lipid free radicals (15). The lipid free radicals react with the Cu^{2+}, reducing it to Cu^{+}. The reduced Cu^{+} is capable of catalyzing further hydroperoxide decomposition. The resulting peroxyl lipid free radicals have the power to oxidize unsaturated lipids in the LDL particle (15). Oxygen-free radicals are also capable of modifying LDL in the absence of cells. Hydroxyl (HO°) and hydroperoxyl (HOO°) free radicals were oxidants of LDL in the treatment medium alone (5, 15).

LDL modified by the BASMC (cell-mediated oxidation) showed an increase in thiobarbituric acid-reacting substances (TBAR) when compared to the auto-oxidation of LDL (Table 1). Exposure of LDL to cultured BASMC caused both a physical and biological alteration of the LDL (15). Cell-modification of LDL caused an increase in its density, and *in vivo*, the oxidized LDL would have been taken up more readily than native LDL by macrophages. For cells to modify LDL, several components were necessary, including, 1) viable cells, 2) oxygen, 3) transition metal ions, and 4) reducing agents (15). In the presence of BASMC, the LDL (which was in solution) was oxidized by trace metals and oxygen-free radicals like in auto-oxidation, but it was also oxidized by cell-derived superoxides (5, 15). Low pH areas, such as endosomes and the surfaces of membranes (plasma membrane), augmented the conversion of $O_2^{\cdot -}$ to $HOO_2^{\cdot}$ by equilibrium, which could lead to greater peroxidation (5).

Once the LDL particle is oxidatively modified, it becomes cytotoxic to a variety of cells, including BASMC (Table 2). The lipid fraction of the oxidized LDL is particularly cytotoxic to BASMC (2, 8). The production of lipid free radicals can cause an increase in cell permeability (15). The presence of oxygen-centered radicals can lead to the fragmentation of cellular proteins in solution, and in lipid environments (such as the cell membrane) (5).

However, when the BASMC incubated with LDL were compared to cells incubated in serum-free (treatment) medium, the BASMC + LDL fared better (Table 2) the first 24 hours of treatment. This can be explained by the fact that lipoproteins are an essential component of the BASMCs' diet (15), and during the first 24 hours the cells were able to use, to a certain degree, the LDL as a food source. The BASMC in the treatment medium alone starved. After 24 hours, however, the LDL became too oxidized for the BASMC, and was cytotoxic (Table 4).

Electrophoresis was performed on samples of LDL modified after treatment to give a visual representation of the extent of LDL oxidation (Figure 6 and Table 5). The alterations to the physiochemical properties of LDL that occurred when it was oxidized caused an increase in the electrophoretic mobility values of the modified LDL (11). The oxidation of polyunsaturated fatty acids was the initiating factor in the modification of LDL. Aldehydes (particularly malondialdehyde, which reacts in the TBAR assay to measure the rate of oxidation) are formed by the decomposition of peroxidized polyunsaturated fatty acids. The aldehydes react with amino groups of proteins and with amino acids to form Schiff bases. The interaction of aldehydes with the lysine of the apoprotein B in LDL changes the electrophoretic mobility of LDL (11). The electrophoresis data and the REM values calculated from it supports the TBAR data for the oxidation (auto and cell-induced), and prevention of oxidation, of LDL.

Ketoconazole's Effects on the Oxidation of LDL

The main anti-fungal action of KC as an anti-fungal drug is thought to be the inhibition of cytochrome P-450 dependent 14α-demethylation of lanosterol (7). In mammalian cells, the lanosterol 14α-demethylation step is necessary for cholesterol biosynthesis, and since KC can bind to mammalian cytochrome P-450, KC can inhibit cholesterol biosynthesis. In humans, the inhibition of cytochrome P-450 by KC can reduce total serum cholesterol approximately 30%. KC is also capable of impairing the intracellular movement of LDL-derived cholesterol from lysosomes to the plasma membrane (16), further lowering serum levels by inhibiting the release of cell-manufactured LDL/cholesterol. Also, by inhibiting cytochrome P-450 enzymes, some of which can release activated O_2 that can then participate in the oxidation of LDL (2,7), KC can further lower the extent of LDL oxidation.

KC is also capable of inhibiting lipid peroxidation in microsomal and liposomal

the membranes of the LDL particle and BASMC (7). Membrane stabilization occurs from an association between the hydrophobic rings of cholesterol and the saturated, monosaturated and polyunsaturated residues of phospholipid fatty acids, which decreases the fluidity of the membrane bilayer. KC is able to exert membrane stabilizing effects, similar to cholesterol, against lipid peroxidation because of its structure. The structure of KC is similar to that of lanosterol, and presumably therefore to cholesterol. Membrane stabilization decreases the extent of oxidation by maintaining the structure of the LDL particle and the BASMC. When the LDL's membrane is intact, its core of cholesterol esters is protected from oxidation. With an intact cell membrane, the BASMC do not release hydrolytic enzymes (which can augment LDL oxidation). By stabilizing membranes, BASMCs' viability is increased in the presence of KC because the extent of LDL oxidation is lowered (the LDL is cytotoxic when it is oxidized). BASMC incubated with LDL and KC survived better than cells that where incubated without KC (Figure 4).

It is believed that KC's main anti-oxidant property is its membrane stabilizing effects (Figure 3). By stabilizing membranes, KC lowers the extent of LDL oxidation (and the formation of cytotoxic, oxidized LDL), and helps to prevent damage to the cells from existing oxidized LDL. Both of these actions help to maintain cell viability. Increased cell viability further lowers the extent of LDL oxidation since there are fewer damaged or dead cells to augment the oxidation process. KC did not appear to have any metal chelating properties (such as butylated hydroxytoluene), which is probably why KC was not able to lower the extent of oxidation below that of auto-oxidation.

CONCLUSIONS

The BASMC line obtained from S. Parthasarathy was able to modify LDL. It was found that these cells were capable of oxidizing LDL, as determined by both TBAR and agarose gel electrophoresis data. The cells oxidized LDL in proportion to the number of cells present (i.e., the higher the initial cell concentration, the greater the extent of

oxidation). It was also found that the oxidized LDL was cytotoxic, as determined by protein or cell viability assays, and that the extent of cytotoxicity was dependent on how oxidized the LDL was, and the number of cells present. Although the higher cell concentrations oxidized LDL to a greater extent, the viability of the cells was better than for the lower cell seeding concentrations. This can be explained by the fact that the cells need to be in contact with one another to survive stressful conditions (such as being in the presence of oxidized LDL), and the lower seeding concentrations did not have enough cells for this to happen.

LDL was oxidized in both the absence (auto-oxidation), and presence (cell-mediated) of BASMC. When the LDL was oxidized, there was an increase in the amount of thiobarbituric acid-reacting substances present, an increase in electrophoretic mobility of the LDL, and a loss in BASMC viability. The addition of KC to the medium containing LDL caused a decrease in the extent of LDL oxidation. In the presence of BASMC, Ketoconazole caused a concentration dependent inhibition of LDL oxidation (the higher the concentration of KC, the lower the extent of LDL oxidation). Although KC was able to lower the extent of cell mediated-oxidation, it was not able to lower the extent of LDL oxidation below that of auto-oxidation. This could indicate that KC's main anti-oxidant properties involve interacting with the cell itself (i.e., maintaining the BASMCs' membranes). BASMC viability was maintained in the presence of KC and LDL because the KC lowered the extent of LDL oxidation, which decreased the amount of cytotoxic, oxidized LDL.

The addition of the serum replacement product Celox was beneficial in maintaining the viability of the BASMC. Experiments were generally conducted in serum-free medium, which resulted in the loss of approximately 50% of the BASMC from serum starvation. When Celox was added to the serum-free medium, the viability of the BASMC was increased. As a result, the extent of cell-mediated oxidation increased due to the higher

number of cells present to oxidize the LDL. It is recommended that further work with this line of BASMC be done in medium containing Celox.

LITERATURE CITED

1. Brown, M. S.; Goldstein, J. L. How LDL receptors influence cholesterol and atherosclerosis. Atherosclerosis. 4 (4): 58-66; 1990.

2. Carew, T. E. Role of biologically modified low-density lipoprotein in atherosclerosis. The American Journal of Cardiology. 64: 18G-22G; 1989.

3. Steinburg, D.; Witztum, J. L. Lipoproteins and atherogensis. Journal of the American Medical Association. 264 (23): 3047-3052; 1990.

4. Bedwell, S.; Roger, D. T.; Jessup, W. The action of defined oxygen-centered free radicals on human low-density lipoprotein. Biochemical Journal. 262: 707-712; 1989.

5. Esterbauer, H.; Striegl, G.; Puhl, H.; Oberreither, S.; Rotheneder, M.; El-Saadani, M.; Jurgens, G. The role of vitamin E and carotenoids in preventing oxidation of low density lipoproteins. Annals New York Academy of Sciences. 46: 254-259; 1987.

6. Heinecke, J. W.; Rosen, H.; Chait, A. Iron and copper promote modification of low density lipoprtoein by human arterial smooth muscle cells in culture. The American Society for Clinical Investigation, Inc. 74: 1890-1894; 1984.

7. Wiseman, H.; Smith, C.; Arnstein, H. R. V.; Halliwell, B.; Cannon, M. The antioxidant action of ketoconazole and related azoles: comparison with tamoxifen and cholesterol. Chemical-Biological Interactions. 79: 229-243; 1991.

8. Morel, D. W.; DiCorleto, P. E.; Chisolm, G. M. Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. Arteriosclerosis. 4 (4): 357-364; 1984.

9. Landegren, U. Measurement of cell numbers by means of the endogenous enzyme hexosaminidase. Journal of Immunological Methodology. 67: 379-388; 1984.

10. Hatch, F. T.; Lees, R. S. Practical methods for plasma lipoprotein analysis. Advances in Lipid Research. 6: 1-68; 1968.

11. VanHinsbergh, V. W. M.; Scheffer, M.; Havekes, L.; Kempen, H. J. M. Role of endothelial cells and their products in the modification of low-density lipoproteins. Biochemica et Biophysica Acta. 876: 49-64; 1986.

12. Robbins, E.; Jentzch, G. Rapid embedding of cell culture monolayers and susupensions for electron micrscopy. Journal of Histochemistry and Cytochemistry. 15: 181; 1967.

13. Postek, M.T., Howard, K. S., Johnson, A. H., and McMichael, K. L. Scanning electron microscopy, a student's handbook.. Ladd Research Industries; 1980.

14. Methods in enzymology, Vol. LVIII. Academic Press, Inc.; 1979.

15. Gebicki, J. M.; Jürgens, G.; Esterbauer, H. Oxidation of low-density lipoprotein in vitro. Oxidative stress: oxidants and antioxidants. Academic Press Ltd., 1991: 371-397.

16. Liscum, L. Pharmaological inhibition of the intracellular transport of low-density lipoprotein-derived cholesterol in chinese hamster ovary cells. Biochemica et Biophysica Acta. 13: 1-9; 1990.